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Familial colorectal cancer and polyposis, genes, pathways and predictions.

Dr Lara R. Lipton

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Colorectal cancer in the commonest internal malignancy in western society today. At least a third of the incidence is likely to be due entirely or in part to inherited genetic factors. Over the last 15 years several genes have been described in which germline mutation leading to increased colorectal cancer risk may occur. The commonest are Hereditary Non-Polyposis Colorectal Cancer (HNPCC), which accounts for around 1-4% of colorectal cancer diagnosis without polyposis and is caused by mutations in mismatch repair genes and Familial Adenomatous Polyposis caused by mutations in the *APC* gene. In this thesis two related themes are addressed. Firstly I examine clinical, pathologic and molecular genetic information in kindreds recruited from family cancer clinics in order to investigate several relevant clinical problems relating to decisions regarding genetic testing and clustering of non-HNPCC families. Secondly, the group of individuals and families with multiple colorectal polyps without known genetic cause are investigated in several ways. A candidate gene analysis is undertaken looking for germline changes, an analysis of adenomas from such individuals for informative somatic changes is performed and I describe a new inherited syndrome of colorectal cancer and polyposis, MYH associated polyposis as well as the pathway of tumourigenesis in affected individuals.

I, Lara Lipton, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Figures

Chapter 1 Introduction

1.1 Colorectal Cancer – Hereditary Predisposition

Colorectal cancer (CRC) is the most common internal malignancy occurring in both men and women in Western society. Incidence is also rising steadily in Asian countries. Inherited and somatic genetic changes as well as environmental factors almost certainly contribute to its occurrence. Lifestyle factors and diseases common to our society such as obesity, lack of exercise, diets low in fruit and vegetables and diabetes all contribute to our high rate of CRC diagnosis.

Resources directed at the management of this major health problem, especially in regards to screening of susceptible persons, are rapidly increasing and one of the major challenges currently is to stratify our screening efforts, with intensive programs using colonoscopy directed towards those at highest risk who will benefit most. It has long been recognised that there are families showing a dominant predisposition to colorectal adenomas and/or cancers, but the identification of the genes and mutations responsible for such increase in familial risk is a much more recent phenomena (Peltokallio and Peltokallio 1966; Bussey 1982; Lynch and Lynch 1985; Lynch, Kimberling et al. 1986). Despite recent advances in the field many individuals and families with strong family histories of colorectal cancer with or without features of polyposis remain without genetic diagnosis. It is thought that about 30% of the total colorectal cancer burden can be accounted for by inherited genetic factors based on calculated relative risks in persons having one or more relatives affected with colon cancer. Relative risks increase when multiple relatives are affected or even one is affected at a very young age (Vasen, Griffioen et al. 1990; Fuchs, Giovannucci et al. 1994; Dunlop 1998; Winawer, Fletcher et al. 2003).

The overall aims of the following work include the categorisation and further analysis of patients and families who appear to have an increased predisposition to colorectal cancer and/or colorectal polyps and who do not possess pathogenic mutations in one of the previously described colorectal cancer predisposition genes. Identifying new genetic factors predisposing to colorectal cancer increases our ability to understand the following-

- Knowledge of specific factors involved in carcinogenesis in both sporadic and inherited forms of cancer can provide targets for diagnostic tests, preventative strategies and novel treatment modalities.

- Identification of germline genetic predisposition by detection of specific gene mutations within a family member allows for the option of predictive testing among other family members. Mutation carriers within families can now be offered effective screening options to reduce the incidence and mortality of colorectal cancer (Jarvinen, Aarnio et al. 2000).

- Genes found to play key roles in hereditary colorectal cancer syndromes are often found to contribute to the pathogenesis of sporadic colorectal carcinogenesis via somatic mutation leading to loss and epigenetic gene silencing of tumour suppressor genes or pathological activation of oncogenes.

1.2 Overview of Hereditary Colorectal Cancer Syndromes

Known inherited colorectal cancer predisposition syndromes can be usefully classified into those with and without large numbers (tens to thousands) of colorectal polyps or polyposis. The former can be further delineated by polyp histology. The non-polyposis colorectal cancer syndromes include Hereditary Non-Polyposis Colorectal Cancer (HNPCC) and its variants Muir-Torre and Turcot's syndromes. These are secondary to germline mutations in one of four mismatch repair (MMR) genes, *MLH1, MSH2, MSH6* and *hPMS2* with *MLH1* and *MSH2* being most commonly found to harbour mutations. Inflammatory syndromes of the colorectum, primarily Crohn's disease and ulcerative colitis (UC) also predispose to cancer. Inheritance may play a part in a proportion of these cases.

Familial adenomatous polyposis (FAP), incorporating Gardner's syndrome and some Turcot's syndrome families, is characterised by in the presence of multiple adenomatous colorectal polyps, whereas in juvenile polyposis syndrome (JPS) and Peutz-Jeghers syndrome (PJS), the tumours are hamartomatous in nature. All are associated with an increased risk of developing colorectal cancer. Adenomas are dysplastic lesions with the proliferating epithelial tubules packed closely together in glands. Epithelial cells show disorganised, hyperchromatic nuclei, an increased nuclear to cytoplasmic ratio and an increased mitotic

rate. Hamartoma are characterised by overgrowth of cells or tissues native to the area in which they normally occur. This typically involves the mesenchymal or stromal components, although the endodermal or ectodermal elements may be involved. Epithelial dysplasia may occur as hamartoma grow with invasive malignancy as a consequence. Hamartomatous polyps are also a feature of Cowden disease (CD) and Bannayan-Zonana Syndrome (BZS) (Hendriks, Verhallen et al. 2003; McGarrity, Wagner Baker et al. 2003; Merg and Howe 2004). In these syndromes an increased risk of intestinal and other organ-specific malignancies is seen (Frayling, Bodmer et al. 1997). The hereditary mixed polyposis syndrome (HMPS) is phenotypically an overlap syndrome characterised by both adenomatous and hamartomatous polyps (Jaeger, Woodford-Richens et al. 2003). Intestinal polyps have also been described in Cronkhite-Canada syndrome and in tuberous sclerosis, but their clinical significance is less certain (Digoy, Tibayan et al. 2000; Jain, Nanda et al. 2003; Nagata, Kijima et al. 2003; Yashiro, Kobayashi et al. 2004).

1.3 Colorectal Carcinogenesis and Genomic Instability

For the analysis of the molecular genetics of CRC I make several hypotheses. Firstly that cancer results via a multistep progression at both the molecular and the morphologic level. Secondly is that loss of stability of the genome is a key step in cancer formation. This may include chromosomal instability or defects in systems maintaining DNA sequence integrity. Thirdly that the genes affected in hereditary cancer syndromes frequently correspond to key genetic defects whose somatic occurrences drive the emergence of sporadic colon cancer. For example, one of the early events in the development of sporadic colorectal cancer is disruption in the *Wingless*/Wnt signaling pathway component *APC* which is also implicated in the syndrome of FAP.

The evolution of normal epithelial cells to adenocarcinoma usually follows a predictable course of histological and genetic changes (Vogelstein, Fearon et al. 1988. The changes provide a growth advantage and lead to clonal expansion of altered cells. Subsequent waves of clonal expansion then occur as a consequence of progressive events that provide other growth advantages to the cell such as loss of cell contact inhibition and the ability to metastasise. The earliest identifiable lesion in colon cancer formation is the unicryptal adenoma (dysplastic changes occurring in a single colonic crypt). It appears that even these

lesions can harbour mutations in KRAS and APC and may progress to cancer (Smith, 1993).

In addition to affecting cell biology, some genetic and epigenetic alterations result in loss of genomic stability, which contributes to the accumulation of mutations in tumour suppressor genes and oncogenes. Both the putative phenomena of chromosomal instability (CIN) and microsatellite instability (MSI) appear to occur early in tumourigenesis (Jacoby, Marshall et al. 1995; Ried, Knutzen et al. 1996) (Shih, Zhou et al. 2001; Nowak, Komarova et al. 2002). The underlying cause for CIN is still unknown, although recent studies have implicated mutations of *APC* as a possible cause. It has recently been shown that in cells with mutant *APC*, karyotypic abnormalities arise, specifically polyploidy, which suggests that APC may play a key role in chromosomal segregation and potentially in chromosomal instability, however, little evidence for APC's role in karyotypic abnormality exists in vivo (Fodde, Kuipers et al. 2001; Fodde, Smits et al. 2001; Kaplan, Burds et al. 2001).

1.4 Familial Adenomatous Polyposis (FAP)

1.4.1 The Syndrome of Adenomatous Colonic Polyposis

Familial Adenomatous Polyposis (FAP) accounts for approximately 1% of all colorectal cancer cases seen in practice. It is has long been viewed as a distinctive syndrome, both in clinical presentation and genetic background (Bussey 1987). Classical FAP affects ~1 in 10,000 individuals and is characterised by the presence of hundreds to thousands of adenomas in the colon and rectum (Bisgaard, Fenger et al. 1994; Bulow, Faurschou Nielsen et al. 1996). This leads to an almost 100% chance of malignant transformation in at least one of these polyps by the fifth decade. Polyps also develop in the upper gastrointestinal tract, especially the duodenum and these will progress to malignancy in approximately 10% of those so affected(Bulow, Alm et al. 1995; Sieber, Lamlum et al. 2002; Bulow, Bjork et al. 2004). Fundic gland polyps also occur but there is no documented increased risk of gastric cancer in this condition. There is an increased risk of malignancy in other sites including the Ampulla of Vater (adenocarcinoma), the brain (glioblastoma), the thyroid gland (papillary carcinoma, especially in young women), and the liver (hepatoblastoma in young children) (Giardiello, Offerhaus et al. 1991; Lynch, Smyrk et al. 1993; Mori, Nagase et al. 1994; Hamilton, Liu et al. 1995; Cetta, Montalto et al. 1997; Soravia, Sugg et al. 1999; Hirschman,

Pollock et al. 2005). Desmoid tumours are benign cancers of the connective tissues that can lead to life-threatening complications through their sheer size and impingement on vital structures (Gurbuz, Giardiello et al. 1994; Heiskanen and Jarvinen 1996; Pikaar, Nortier et al. 2002). They occur in 5 to 10% of FAP patients, more commonly occurring in women. Other diagnostic features of FAP include retinal lesions, known as congenital hypertrophy of the retinal pigment epithelium (CHRPE), found in 60-90% of FAP patients, epidermis cysts, most notably on the scalp, osteomas (benign tumours of bone) and dental anomalies (e.g supernumerary and unerrupted teeth) in about one third of patients (Reck, Bunyan et al. 1997; Tourino, Conde-Freire et al. 2004; Bisgaard and Bulow 2006). Inheritance is autosomal dominant although 30% of cases do not have a family history of polyposis. In some of these persons a new *APC* mutation in the individual is causative and in others it is likely that separate genetic causes are responsible. This leads to a seemingly recessive phenotype within the family (Bisgaard, Fenger et al. 1994).

In undiagnosed cases of FAP, the leading cause of death is colorectal cancer. If diagnosed in childhood or early adulthood and managed appropriately, early death can still occur, principally from duodenal cancer and the effects of desmoid tumours (Galle, Juel et al. 1999). FAP shows variable clinical expression and severity between different families, some of which is explained by the location of the germline mutation within the gene (Nagase, Miyoshi et al. 1992; Giardiello, Petersen et al. 1997; Crabtree, Tomlinson et al. 2002). Different families display different combinations of extracolonic features. Previously families with prominent extracolonic features, including sebaceous cysts, osteomas, desmoid tumours and dental abnormalities were classified as having Gardner's Syndrome. This is now known to be a phenotypic variant of FAP (Leppert, Dobbs et al. 1987; Nakamura, Nishisho et al. 1991; Nishisho, Nakamura et al. 1991; Nakamura, Nishisho et al. 1992; Giardiello, Petersen et al. 1997; Griffioen, Bus et al. 1998). Management of this condition will be discussed later.

1.4.2 The Adenomatous Polyposis Coli Tumour Suppressor Gene

The identification of an interstitial deletion on Chromosome 5q in a patient with Gardner's syndrome combined with linkage analysis facilitated the positional cloning of the gene for FAP, *Adenomatous Polyposis Coli (APC)* in 1991 (Leppert, Dobbs et al. 1987; Groden, Thliveris et al. 1991; Kinzler, Nilbert et al. 1991; Kinzler, Nilbert et al. 1991; Nakamura, Nishisho et al. 1991; Nishisho, Nakamura et al. 1991). This gene has since been shown to

play an integral role in the Wnt signalling pathway, especially in regard to the degradation of β-catenin within the cell cytoplasm (Rubinfeld, Albert et al. 1997) (Munemitsu, Albert et al. 1995). The gene has 15 exons with exon 15 being a giant exon, and encodes a protein of up to 2861 amino acids (310kDa). Germline mutations in *APC* have been found to be responsible for 70-90% of FAP cases and families. The APC protein occurs in multiple isoforms within cells, probably a result of alternative splicing at the mRNA level (Sulekova, Reina-Sanchez et al. 1995). The most common transcript lacks exon 10a and the protein contains 2843 amino acids. APC is thought to perform a number of intracellular functions and contains several amino acid motifs and domains, allowing it to dimerise and to interact with numerous other molecules having diverse functions with in the cell. B-catenin, GSK3β, axin, conductin, tubulin, EBI and hDLG are the major ones identified (Rubinfeld, Souza et al. 1993; Su, Burrell et al. 1995; Deka, Kuhlmann et al. 1998; Sparks, Morin et al. 1998; Ikeda, Kishida et al. 2000; Spink, Polakis et al. 2000). Two motifs within the gene interact with β-catenin, a set of three x 15 amino acid repeats between residues 1020 and 1169 and a repeating sequence of seven x 20 amino acid repeats between residues 1262 and 2033 (Rubinfeld, Souza et al. 1993; Rubinfeld, Albert et al. 1997). These motifs are highly conserved across species. Each 20 amino acid repeat contains a site mediating glycogen synthase kinase 3β (GSK3β), binding. Indispersed between the 20 amino acid repeat sequences are three SAMP (Ser-Ala-Met-Pro) repeats which are sites of axin binding. A region between 2200 and 2400 amino acids mediates microtubule binding and areas closer to the C-terminus (residues 2560-2843) interact with the microtubule associated protein EB1.

1.4.3 *APC* Gene Functions

The major known function of APC within the Wnt/wingless pathway is a negative regulator of β-catenin (Hayashi, Rubinfeld et al. 1997; Hart, de los Santos et al. 1998; Chan, Wang et al. 2002). Upon binding of Wnt-1 to its transmembrane receptor frizzled, the disheveled protein is activated and inhibits the serine-threonine kinase GSK3β (Ikeda, Kishida et al. 2000) (Rubinfeld, Albert et al. 1997). This represses β-catenin degradation and there is resulting accumulation of β-catenin, which can then translocate to the nucleus and interact with other transcription factors like T-cell factor (TFC) (Roura, Martinez et al. 2003; Hamada and Bienz 2004; Haraguchi, Nishida et al. 2004). TCF-4 is the predominant TCF family member expressed in colonic epithelium. Relevant targets upregulated by TCF-4 identified to date include cyclin D1, c-myc, matrilysin, c-jun,fra-1, MDR-1 (multidrug resistance 1

gene), GAS (gastrin gene), vascular endothelial growth factor and urokinase-type plasminogen activator receptor (Castrop, van Wichen et al. 1995; Korinek, Barker et al. 1997; He, Sparks et al. 1998; He, Chan et al. 1999; Roose and Clevers 1999; Clements, Lowy et al. 2003; Easwaran, Lee et al. 2003). Overexpression of Wnt targets cyclin D1 and c-myc, has been demonstrated in early adenomas from FAP patients (He, Sparks et al. 1998) (He, Sparks et al. 1998). APC is phosphorylated by GSK3β and complexes with the proteins axin and conductin. This complex mediates the ubiquination and degradation of β-catenin within the proteosome. The importance of APC-mediated β-catenin degradation is highlighted by the location of the mutation cluster region within the region of the *APC* gene encoding the 20-aa repeats. Activating mutations of β-catenin in the GSK3β phosphorylation site required for its degradation results I the accumulation of β-catenin and have been documented in approximately 50% of colorectal tumours that do not contain *APC* mutations (Morin, Sparks et al. 1997)Experiments demonstrate that the nuclei of colon carcinoma cells not expressing APC contain stable β-catenin/Tcf4 complexes, which are constituently active, as measured by transcription of a TCF reporter gene (Korinek, Barker et al. 1998). In one experimental study, the introduction of *APC* removed β-catenin from TCF-4 and transcriptional activation ceased (Korinek, Barker et al. 1997). Evidence exists that APC is able to shuttle between nucleus and cytoplasm within the cell, possibly allowing it to compete with TCF-4 FOR β-catenin binding and remove β-catenin to the cytoplasm for degradation (Rosin-Arbesfeld, Townsley et al. 2000).

Other functions for APC include a role in cytoskeletal organisation. APC binds to the microtubule cytoskeleton via its basic domain and has a role in cell migration, division and polarity (Mimori-Kiyosue, Shiina et al. 2000; Mimori-Kiyosue, Shiina et al. 2000). The microtubule binding function of APC may also contribute to proper chromosome segregation during mitosis (Fodde, Kuipers et al. 2001; Kaplan, Burds et al. 2001). APC is also thought to play a pro-apoptotic role. Studies have shown that in SW480 cells which normally express truncated APC protein, delivery of full length APC promotes G1 cell cycle arrest and apoptosis (Groden, Joslyn et al. 1995).

1.4.4 Germline Mutations in the *APC* Gene

Germline mutations in the *APC* gene cause FAP, and somatic mutations occur in FAP tumours and sporadic colorectal tumours. Germline mutations throughout the *APC* gene

differ in their penetrance, severity of polyposis and the expression of extra-colonic features (Bodmer 1999). About 95% of *APC* germ-line mutations are either nonsense (28%) or frameshifts (67%) and hence the protein truncation test is generally adequate for their detection (Kinzler, Nilbert et al. 1991; Nagase, Miyoshi et al. 1992; Smith, Johnson et al. 1993). Genotype-phenotype correlations exist with regard to the location of the germ-line mutation within the gene (Wallis, Morton et al. 1999). Generally, mutations in the central region of the gene (codons 1250-1464) give a profuse polyposis phenotype with thousands of intestinal polyps (Classical FAP). Two codons, 1061 and 1309 are mutational hotspots and account for approximately 11% and 17% of all germline mutations (Groden, Gelbert et al. 1993). CHRPE is associated with germline mutations between codons 1395 and 1560 (Wallis, Macdonald et al. 1994) . A specific syndrome historically, Turcot's syndrome, described as the occurrence within families of primary brain tumours (medulloblastoma) and colorectal adenomas, was shown in 1995 to be principally due to mutations in the *APC* gene (Lasser, DeVivo et al. 1994; Mori, Nagase et al. 1994; Hamilton, Liu et al. 1995; Rutz and Kikuchi 1995; Kunikata, Abe et al. 2000). These mutations, though all truncating, were heterogenous in type and location. A smaller number of phenotypically similar families whose primary brain tumours are glioblastoma multiforme have disease caused by various mutations in the known MMR genes (Leung, Chan et al. 1998; Chan, Yuen et al. 1999).

1.4.5 Germline *APC* Mutations in Attenuated Adenomatous Polyposis Coli

The existence of an attenuated form of FAP had been suggested in 1990 by Leppert who showed linkage to 5q in a large family with an attenuated polyposis phenotype (Leppert, Dobbs et al. 1987). Families with attenuated adenomatous polyposis coli (AAPC) are characterised by the presence of one or more mutation carriers with multiple colorectal adenomas (<100) rather than profuse colorectal polyposis. Average age of diagnosis within families is later than that of classical FAP with cancer occurring, on average, 15 years later (Lamlum, Al Tassan et al. 2000; Sieber, Segditsas et al. 2006). Variable penetrance is seen as well as highly variable phenotype within families. Within a single AAPC kindred it is not uncommon to see mutation carriers with few polyps and others with a classic FAP phenotype. Overall extra-colonic features are less common but may occur in some mutation carriers. AAPC is associated with germline mutations occurring in the 5' (codons 78-167) and 3' (codons 1581 to 2843) regions of the *APC* gene and also in exon 9 (Spirio, Otterud et al. 1992; Spirio, Olschwang et al. 1993; van der Luijt, Meera Khan et al. 1996; Spirio, Green

et al. 1999) (Soravia, Berk et al. 1998). An attenuated phenotype has also been reported in some families with a complete deletion of one copy of the *APC* gene (Hodgson, Coonar et al. 1993; Hodgson, Fagg et al. 1994). The latter is, however, likely to be a rare occurrence (Sieber, Lamlum et al. 2002). Some mutations associated with the AAPC phenotype lead to an unstable mRNA or protein. Other mutations in alternately spliced exons, such as those in exon 9, are spliced out of at least some mRNA species, resulting in a nearly full-length protein lacking the exon carrying the mutation (van der Luijt, Vasen et al. 1995; Young, Simms et al. 1998). It has been proposed that for tumours to occur in these patients both the wild type and the germline variant alleles need to undergo mutations or loss. Other genetic and environmental factors also influence clinical expression, as individuals with identical *APC* germline mutations are known to develop dissimilar phenotypes within and between families (Crabtree, Tomlinson et al. 2002) (Brensinger, Laken et al. 1998; Soravia, Berk et al. 1998). Sibling pairs show a much higher correlation in disease severity and polyp number than do parent-child pairs or any other more distant relationship. Within AAPC families polyp numbers may be highly variable with some mutation carriers developing hundreds of polyps and others very few (van der Luijt, Meera Khan et al. 1996; Soravia, Berk et al. 1998).

AAPC or the multiple adenoma (MA) phenotype is due to detectable mutations in the *APC* gene in only a small proportion of cases (approximately 5%) (Lamlum, Al Tassan et al. 2000; Sieber, Lamlum et al. 2002).

1.4.6 Germline Missense Variants in *APC*

Two important missense variants in *APC* have been reported. The 11307K variant occurs in about 9% of Ashkenazi Jews and 2% of non-Ashkenazi Jews but is very rare outside this ethnic group with rates of 0 to 2% depending on ethnicity (Laken, Petersen et al. 1997; Guo, Lim et al. 2004; Kapitanovic, Cacev et al. 2004). It is present in around 10% to 12% of Ashkenazi jewish patients with colorectal cancer (Fidder, Figer et al. 2005; Rennert, Almog et al. 2005; Zauber, Sabbath-Solitare et al. 2005). Estimates of 10 to 28% risk of colorectal cancer exist for Ashkenazim with a family history of colorectal cancer and who carry the mutstion (Drucker, Shpilberg et al. 2000; Strul, Barenboim et al. 2003H). The relative risk of colorectal cancers in I1307K carriers may be around 1.5(Frayling, Beck et al. 1998; Gryfe, Di Nicola et al. 1998; Strul, Barenboim et al. 2003) (Rozen, Shomrat et al. 1999). Other studies

have found no increase in CRC risk (Woodage, King et al. 1998). The question of colorectal screening for I1307K carriers remains open to debate. As colorectal cancer risk is only mildly elevated, the majority of centres do not advocate increased surveillance compared to the average population. Some centres in Israel suggest colonoscopy five to ten yearly for carriers (Rozen, Naiman et al. 2002).

The I1307K mutation leads, not to a truncated protein, but to the formation of an A8 tract (from the normal A3TA4) that is hypermutable and may undergo somatic slippage in colonic tissue to produce somatic frameshift mutations (Gryfe, Di Nicola et al. 1998). The 11307K allele frequently undergoes somatic mutation or is lost in tumour DNA suggesting that it does not directly participate in the tumourigenic process (Sieber, Lipton et al. 2003; Sieber, Heinimann et al. 2003). Studies of somatic *APC* mutations in cancers from I1307K patients commonly show a second mutation in the *APC* allele carrying I1307K . However as this missense mutation occurs near a site where other germline mutations cause very severe disease (for example truncating mutations at codon 1309), other functional effects of this mutation cannot be ruled out.

A further, missense variant E1317Q has been associated with the AAPC phenotype but has no clear effects on hypermutability (Frayling, Beck et al. 1998; Popat, Stone et al. 2000; Gismondi, Bonelli et al. 2002; Hahnloser, Petersen et al. 2003). It is present in around 0.5 to 2% of the population and studies show little convincing evidence of a role in colorectal adenoma or cancer predisposition (Lamlum, Al Tassan et al. 2000; Gismondi, Bonelli et al. 2002). E1317Q codes for a mutation in *APC* in the MCR that lies between the first and the second 20-amino acid β-catenin binding site. Limited evidence exists for an affect of this variant on APC function in respect to the degradation of β-catenin pathway. No special colorectal screening is currently recommended for carriers.

1.4.7 Somatic Mutations in *APC*

APC is a tumour suppressor gene, generally requiring the loss or mutation of both gene copies for tumour initiation. In the tumours occurring in germline *APC* mutation carriers, the wild-type allele is generally disrupted by another mutation or, less frequently, lost. Most sporadic colorectal cancers also carry two inactivating *APC* mutations (Nakamura, Nishisho et al. 1992; Fodde 2002). In both familial and sporadic cases, somatic mutations are mainly

confined to the 5' half of the gene. The range is smaller than that for germline mutations generally, with over 80% of somatic mutations occurring between codons 1284 and 1580, the mutation cluster region (MCR) (Nakamura, Nishisho et al. 1992; Nagase and Nakamura 1993; Miyaki, Konishi et al. 1994; Crabtree, Sieber et al. 2003). The MCR contains a set of 20 aa repeats thought to be crucial for beta-catenin binding and degradation (Rubinfeld, Albert et al. 1997). Mutations at these positions suggest that loss or alteration of β-catenin binding is a crucial step towards tumour formation. These mutations are often accompanied by loss of the residual wild-type allele. Three somatic mutational hotspots occur at codons 1309, 1450 and 1554 accounting for approximately 75, 8% and 5% of somatic mutations respectively (Rowan, Lamlum et al. 2000). In *APC* most point mutations are C to T transitions, at CGA motifs – generating the stop codon TGA (Nakamura, Nishisho et al. 1992).

An association is now known to exist between the positions and types of the first and the second inactivating mutations in *APC* both FAP and sporadic tumours. Initial mutations around codon 1300 are associated with loss of wild type *APC* allele. In contrast, patients with germline mutations 3' and 5' to this generally show truncating 'second hits' in the mutation cluster region (Lamlum, Ilyas et al. 1999; Albuquerque, Breukel et al. 2002; Cheadle, Krawczak et al. 2002). Mutations in the different areas of the gene probably have different selective advantages to tumour growth with those in the MCR, around 1300 providing the strongest impetus to tumour growth. Hence FAP patients with germline *APC* mutations around codon 1300 already possess the most strongly selected mutation and their adenomas lose the wild-type as a 'second hit' (Crabtree, Sieber et al. 2003). Patients with a germline mutation outside this region need to acquire a truncating mutation within the MCR to select for tumour growth. Sometimes the germline mutant in such patients is lost in the tumours of such patients in a 'third hit', resulting in the advantageous combination of one truncating mutation in the MCR and one lost allele (Spirio, Samowitz et al. 1998; Sieber, Segditsas et al. 2006).

1.4.8 The *APC* Tumour Suppressor Gene and Tumourigenesis

There is probably no single reason why mutations in *APC* lead to tumour formation. It does not act as a simple tumour suppressor with a second hit causing complete loss of function as in Knudson's hypothesis. The relation between the site and type of first, second and even

third hits, the low frequency of allelic loss at *APC* and the attenuated phenotype that may result from mutations in exons one to four causing highly truncated protein suggest that such loss of function of *APC* may not make a cell as 'cancer-prone' as certain mutations leading to beta-catenin mediated cellular proliferation. Such mutations may give an optimal level of beta-catenin within the cell nucleus for progression to a cancer phenotype (Rubinfeld, Albert et al. 1997; Sieber, Heinimann et al. 2002; Crabtree, Sieber et al. 2003). Mutations giving a more stable truncated protein may also be selected. Like *APC* mutations, *β-catenin* mutations have an essential role in early colorectal tumour formation. The incidence of β-catenin mutations decreases from 2.5% in benign adenomas to 1.4% in invasive cancers suggesting that there is a level of excess β-catenin that does not favour progression from benign adenoma to cancer (Rubinfeld, Robbins et al. 1997; Samowitz, Powers et al. 1999). In support of this, studies of β-catenin overexpression in colorectal adenomas show a gradient of staining from the base to the mouth of the crypt. Strong aberrant nuclear staining at the base gives way to normal membrane staining as the dysplastic cells move up the crypt (Brabletz, Herrmann et al. 2000; Preston, Wong et al. 2003) (Brabletz, Jung et al. 2001).

Mutations in *APC* cause loss of SAMP repeats and C-terminal functions. Truncation of the C terminus of *APC* will disrupt its interaction with EB1. Eb1 belongs to a large family of highly conserved proteins which participate in chromosome segregation, spindle orientation and microtubule integrity (Berrueta, Kraeft et al. 1998; Tirnauer, Canman et al. 2002). APC is seen to localise to the kinetochore of dividing cells, truncating mutations may therefore result in disturbances of mitotic spindle stability and structural chromosomal abnormalities may follow. It is uncertain at what stage of tumour development, chromosomal instability caused by a truncated APC protein may come in to play relative to the effects of *APC* mutations on β-catenin build-up within cells.

1.5 Other Polyposis Syndromes Predisposing To Colorectal Cancer

1.5.1 Peutz-Jeghers Syndrome

The original descrition of Peutz-Jeghers Syndrome is credited to Peutz in 1921. This is an autosomal dominant inherited syndrome of specific hamartomatous polyps and characteristic mucocutaneous pigmentation. It occurs in approximately 1:200,000 live births . Pigmentation is mostly (94%) around lips and buccal mucosa but can also occur in other

areas. It begins to fade at puberty with only buccal pigmentation in adult life. Polyps are benign overgrowths of muscular tissue in the bowel wall, they range in size from millimetres to centimeters and are most common in the small bowel, also occurring in the colorectum and stomach. They are distinct from the polyps of Juvenile Polyposis without the cystic filled spaces common in the latter. Adenomatous change is uncommon but can occur (Hizawa, Iida et al. 1993; Bosman 1999). Disease generally presents with benign complications of polyps, bleeding, obstruction and intussusception in the first three decades and with cancers in later life. Cancer risk includes a variety of sites. By 65 years of age there is a 39% risk of CRC, 36% risk of pancreatic cancer, 29% risk of stomach cancer, 54% risk of breast cancer, 21% risk of ovarian cancer and lower (but still much above population) risks of small bowel, oesophagus, endometrial, malignant sex cord tumour, Sertoli cell tumour and lung cancers (Spigelman, Murday et al. 1989; Hizawa, Iida et al. 1993; Spigelman, Arese et al. 1995; Giardiello, Brensinger et al. 2000). Screening protocols are empiric and include 2 yearly colonoscopy and gastroscopy from late teens, 2 yearly mammography from 35 and annual, pelvic and testicular examinations and ultrasound of abdomen and pelvis, testes in males and ovaries in females (Spigelman and Phillips 1989; McGrath and Spigelman 2001; Dunlop 2002). Women should begin yearly breast examination by a breast surgeon or general practitioner from age 25 (Altaha, Reed et al. 2003; Thull and Vogel 2004).

Disease causing mutations and deletions in the *LKB1/STKII* (19p13) gene have been found in about half of affected families (Hemminki 1999; Wang, Churchman et al. 1999) (Wang, Ellis et al. 1999; Aretz, Stienen et al. 2005). This gene codes for a multifunctional serine-threonine kinase, important in second messenger signal transduction. Most documented mutations occur in the kinase domain of the gene (Scott, Crooks et al. 2002; Boudeau, Baas et al. 2003). Unlike other polyposis syndromes, inactivation of LKB1 occurs in epithelial tissues only (van der Weyden, Jonkers et al. 2002). This contrasts with the Juvenile Polyposis Syndrome, in which, inactivation of *SMAD4* occurs in stromal tissues also. Both conditions result in the formation of hamartoma which consist primarily of stromal elements. Cancerous change, however, when it occurs involves epithelial cells.

1.5.2 Juvenile Polyposis Syndrome (JPS)

Juvenile polyps occur in 2% of children. They are hamatomatous polyps consisting of an overgrowth of lamina propria with mucin retention cysts. They have a smooth surface

covered in exudate. Juvenile Polyposis Syndrome was first described in 1964 (McColl, Busxey et al. 1964). It is the most common of the hamartomatous syndromes and is inherited in an autosomal dominant manner with variable penetrance and with 20 to 50% of cases having a family history of juvenile polyposis (Desai, Neale et al. 1995). The diagnosis of JPS is made with the presence of three or more juvenile polyps in the GI tract, polyposis involving the entire gastrointestinal tract or any number of polyps in a proband with a known family history of juvenile polyposis (Giardiello, Hamilton et al. 1991). Polyps persist into adulthood and, unlike sporadic juvenile polyps, continue to develop. In addition to colorectal cancer, gastric, duodenal and pancreatic cancers have been reported in JPS (Coburn, Pricolo et al. 1995). At least one third of the affected families are found to have disease-causing mutations of the SMAD4 gene on chromosome 18q21, another 30% have mutations of the BMPR1A gene (Howe, Roth et al. 1998; Bevan, Woodford-Richens et al. 1999; Woodford-Richens, Rowan et al. 2001; Zhou, Woodford-Richens et al. 2001; Sayed, Ahmed et al. 2002; Howe, Sayed et al. 2004). In infancy, patients present with gastrointestinal bleeding, either acute or chronic, intussusception, rectal prolapse or protein losing enteropathy. In adulthood these patients will more commonly present with gastrointestinal blood loss and have tens to hundreds of polyps, with a distal colonic distribution. The lifetime risk of colon cancer is about 50% although quoted incidences vary markedly (Coburn, Pricolo et al. 1995; Desai, Neale et al. 1995) (Jass, Williams et al. 1988). A range of congenital defects has been described in association with the non-familial form of the disease. Colonoscopy and gastroscopy are recommended two to three yearly from the teenage years (Burt, Bishop et al. 1990; Dunlop 2002; Half and Bresalier 2004).

1.5.2 Cronkhite-Canada Syndrome

Cronkhite-Canada syndrome is an acquired condition characterised by the rapid onset of generalised gastrointestinal juvenile polyposis (sessile polyps), cutaneous hyperpigmentation, hair loss, nail atrophy, hypogeusia, diarrhoea and weight loss (Johnson, Soergel et al. 1972; Jain, Nanda et al. 2003; Nagata, Kijima et al. 2003; Yashiro, Kobayashi et al. 2004). It is a rare sporadic condition (no familial cases described) with a 12% incidence of colorectal cancer and a course which may be acute and sometimes fatal or protracted. The cause remains elusive.

1.5.2 Cowden Syndrome

Cowden syndrome was first described in 1963 ((Lloyd and Dennis 1963). It is an autosomal dominant condition with hamartomas and mucocutaneous features. It arises through germline mutations in the PTEN gene on chromosome 10q22 in 80% of patients meeting diagnostic criteria (Nelen, Padberg et al. 1996; Liaw, Marsh et al. 1997). Hamartomas involve the skin, intestine, breast and thyroid gland (Eng 2003). Gastrointestinal lesions are mostly juvenile polyps although other types occur. The most frequent skin lesions are facial trichilemmomas, with café-au-lait spots, vitiligo, squamous and basal cell carcinoma also occurring. Oral mucosal lesions, morphologically similar to the trichilemmomas occur in about 85% of patients. Two-thirds of patients develop goitre and there is a 10% risk of thyroid cancer (Eng 1999). Three quarters of affected females develop benign breast disease including fibrocystic disease and fibroadenomas. There is a 50% risk of breast cancer with frequent bilateral occurrence and median age of onset at 41 years (Ball, Arolker et al. 2001; Figer, Kaplan et al. 2002). Additional benign soft tissue and visceral tumours are observed along with developmental abnormalities such as hypoplastic mandible, prominent forehead and high-arched palate (Eng 2003). There is no documented increase in risk of gastrointestinal malignancy.

Related syndromes, Bannayan-Ruvalcaba-Riley syndrome and Lhermitte-Duclos syndrome are also believed to be due to mutation in the PTEN gene (Marsh, Dahia et al. 1997; Perez-Nunez, Lagares et al. 2004). The former is characterised by macrocephaly, lipomas and pigmented macules of the glans penis in addition to other features of Cowden's syndrome, the latter by cerebellar gangliocytomatosis (Zigman, Lavine et al. 1997; Marsh, Kum et al. 1999; Hendriks, Verhallen et al. 2003) (Ruvalcaba, Myhre et al. 1980; Gorlin, Cohen et al. 1992).

1.5.3 The Hyperplastic Polyposis Syndrome

Hyperplastic polyposis is a rare syndrome usually defined as the presence of multiple hyperplastic polyps or the presence of large or proximal hyperplastic polyps. The WHO criteria for hyperplastic polyposis are as follows -
(i). At least five histologicaly diagnosed hyperplastic polyps proximal to the sigmoid colon, of which two are greater than 10mm in diameter, or

(ii) Any number of hyperplastic polyps occurring proximal to the sigmoid colon in an individual who has a first degree relative with hyperplastic polyposis, or
(iii) Greater than 30 hyperplastic polyps but distributed throughout the colon.

Hyperplastic polyposis generally appears to occur in a sporadic manner although families with dominant inheritance have been reported in rare cases (Jeevaratnam, Cottier et al. 1996; Rashid, Houlihan et al. 2000; Lage, Cravo et al. 2004). These patients have an increased risk of colorectal cancer and a higher than normal incidence of CRC among family members (Place and Simmang 1999; Leggett, Devereaux et al. 2001; Hyman, Anderson et al. 2004; Lage, Cravo et al. 2004). This risk may be an over-estimate as many cases (at least 35%) are ascertained as the results of a CRC being diagnosed. No specific extracolonic features have been reported. Large hyperplastic polyps are more likely to be proximal. Hyperplastic polyps show overgrowth of the colonic epithelium with a 'saw-tooth' pattern in colonic crypts. They grow out on stalks like adenomas but do not have nay features of epithelial dysplasia or tissue invasion. Serrated adenomas have a similar morphologic appearance but show dysplasia in epithelial cells. About 3% of polyps in patients with HPP are MSI-H. It has been postulated that hyperplastic polyps and serrated adenomas form the precursors to MSI-H sporadic cancer (Iino, Jass et al. 1999; Jass, Iino et al. 2000; Leggett, Devereaux et al. 2001). It is difficult to prove that this is actually the case. Several cases have been described in which cancer is seen arising form a hyperplastic polyp which does not show MSI in polyp or cancer but rather evidence of chromosomal instability or allelic losses (Hawkins, Gorman et al. 2000). Cancer arising in a hyperplastic polyp is not a common occurrence but does occur. Loss of *MLH1* expression through hypermethylation has been seen in some hyperplastic polyps but this does not appear to be a common cause of sporadic or familial forms of hyperplastic polyposis. Another gene named HPP1 which is predicted to code for a transmembrane protein containing EGF-like domains has been found to be frequently inactivated by methylation in colorectal cancers and polyps including large hyperplastic polyps indicating a possible role in formation or progression of these lesions (Young, Biden et al. 2001). The cause of these syndromes remains obscure. Perhaps a more interesting question may be whether a syndrome exists in which hyperplastic and adenomatous polyps both occur and hyperplastic polyposis is at the extreme end of such presentations. If a germline predisposition is, in fact, responsible for hyperplastic polyposis, it is likely to be recessive in nature as the majority of affected individuals have no family history of polyposis although colorectal cancer may have occurred in the parents.

1.5.6 The Hereditary Mixed Polyposis Syndrome or CRAC 1

Two large Ashkenazi families have been described in which susceptibility to colorectal cancers and adenomas, serrated adenomas, and juvenile polyps segregates in a dominant fashion (Whitelaw, Murday et al. 1997; Tomlinson, Rahman et al. 1999). Genome wide search revealed linkage for these families to a locus on chromosome 15q13-q14 (Jaeger, Woodford-Richens et al. 2003) (Jaeger, Woodford-Richens et al. 2003). 15q13-q14 has been termed colorectal adenoma and carcinoma 1 (CRAC1) or HMPS. All affected individuals share an identical haplotype in this region and penetrance appears high (18 out of 20 with the disease-related haplotype are affected). The specific haplotype in the families reported is most likely derived from a common founder. The minimal region covers a region of 10 cM and the disease-related haplotype has not been found in non-Ashkenazi patients or controls.

1.6 Hereditary Non-Polyposis Colorectal Cancer

1.6.2 The Hereditary Non-Polyposis Colorectal Cancer Syndrome (Lynch Syndrome)

The Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC) is an autosomal dominant condition which accounts for between 2 and 5% of bowel cancer in Western countries (Lynch, Lemon et al. 1997). The syndrome had been described long before the causative genes were identified and the diagnostic criteria known as the Amsterdam criteria had been formulated (Table 1.1). An early report by Warthin described several different families with dominant inheritance of a spectrum of HNPCC cancers and Lynch later continued following up some of these families (Vasen, Offerhaus et al. 1990). The members of affected families have no benign manifestations to help assign affection status, unlike FAP, and the most clearly distinguishing features are young age of colorectal cancer, a dominant pattern of inheritance, and a high frequency of certain other cancers in the family – endometrium, stomach, ovary, small intestine, urinary tract and biliary system (Aarnio, Sankila et al. 1999; Lynch 1999) . Another common event is the development of synchronous or metachronous cancers in individuals. Cancers are commonly in the proximal colon, although a proportion (around 20-30%) occur in descending colon or rectosigmoid (Lee, Petrelli et al. 2001). Compared to sporadic CRC, HNPCC cancers are more often poorly differentiated, mucinous or of signet cell type and show a pronounced lymphoid reaction

(also known as a Crohn's-like reaction) within cancers (Jass 2000; Jass 2004). The progression of precursor lesion or adenoma to carcinoma appears to be more rapid than in sporadic cancers although affected individuals do not develop a marked excess of adenomas. It is feared that interval cancers may occur if colonoscopy is not performed annually or biennially although data to substantiate this approach is difficult to obtain. It is unlikely that a randomized study will ever be performed. Several studies have suggested that, stage for stage, the survival of colorectal cancer patients with this syndrome may be superior to that of non-HNPCC colorectal cancer patients (Sankila, Aaltonen et al. 1996; Percesepe, Benatti et al. 1997; Aarnio, Sankila et al. 1999; Tomoda, Baba et al. 1999). This may well be a function of MSI in the cancer DNA as this same survival benefit is seen for sporadic MSI-H cancers (Ribic, Sargent et al. 2003; Benatti, Gafa et al. 2005).

There have been several attempts to provide a sensitive and specific definition of the syndrome based on clinical features. In 1990 the Amsterdam criteria were established by the International Collaborative Group on HNPCC (ICG-HNPCC) as being minimum criteria for HNPCC in a family, especially in the context of entering such families in genetic trials (Vasen, Mecklin et al. 1991), These criteria state that to diagnose a family as having HNPCC there must be at least three members affected in two or more successive generations, with at least one affected by colorectal cancer younger than 50 years and one being a first degree relative of the other two. The Amsterdam criteria (Table 1.2) whilst still very specific for the presence of a pathogenic MMR gene mutation are overly restrictive, especially given small modern family sizes and the incidence of extracolonic cancers, particularly of the endometrium, in families carrying HNPCC mutations. Modified Amsterdam criteria were published in 1999 after discussions by the ICG-HNPCC in 1998 allowing the inclusion of certain HNPCC related cancers, endometrial, small bowel, ureter or renal pelvis (Table 1.3) (Vasen, Watson et al. 1999). Families without colorectal cancers such as those with endometrial cancers only could thus be classified as having HNPCC. A National Cancer Institute (NCI) workshop in 1997 reviewed molecular, genetic and histological data on HNPCC in order to increase the sensitivity of diagnostic criteria (Rodriguez-Bigas, Boland et al. 1997). They decided upon a set of clinicopathological criteria, which could help identify additional HNPCC patients and families – the Bethesda criteria (Table 1.4). In 2004 a revision of these guidelines was published (Table 1.5) (Umar, Boland et al. 2004). The revised guidelines stress the importance of MSI-H tumour morphology and broaden the spectrum of HNPCC-associated tumours, including pancreas, brain, ovarian and biliary tract

cancers as well as keratoacanthomas and sebaceous adenomas. Notably these guidelines are not designed or intended to be criteria for germline MMR gene analysis or HNPCC diagnosis, but for the performance of microsatellite instability testing and immunohistochemistry for mismatch repair protein expression. The sensitivity of the original Amsterdam criteria for mutation positive families is approximately 61% (specificity 67%) and the modified criteria 72% (Syngal, Fox et al. 2000). If the Bethesda criteria are used to guide mutation testing, sensitivity rises to 94% whilst specificity falls to 25%.

Once a history suggestive of HNPCC has been ascertained, further clues as to the mutation status of the family can be derived from looking for microsatellite instability (MSI) and loss of MMR protein expression within the tumour DNA (discussed later). In families with a consistent family history, microsatellite unstable (MSI-H) tumour DNA and loss of MMR protein expression in tumour, mutation analysis (including deletion studies) of the MMR genes in germline DNA will reveal pathogenic mutations in about 67% of cases (Chaves, Cruz et al. 2000; Loukola, Eklin et al. 2001; Terdiman, Gum et al. 2001). There remain a significant proportion of families who either fit all these criteria but do not have a germline mutation identified or who fulfill Amsterdam criteria without MSI in their tumours and in whom germline mutations are almost never identified. It is therefore likely that other genetic predispositions to colorectal cancer exist, causing tumour formation by differing genetic means.

Without or without the identification of a germline MMR mutation, families need to be counseled and risk management strategies put in place so as to reduce the morbidity and mortality from colorectal, endometrial and other cancers (Menko, Wijnen et al. 1996; Beck, Tomlinson et al. 1997; Lynch, Watson et al. 1999). Once a definite pathogenic germline mutation has been identified within a proband, predictive testing is possible for unaffected family members. Predictive testing is carried out after a programme of counseling, considering the consequences of both a positive and a negative test to each individual (Lerman, Hughes et al. 1999; Lynch, Watson et al. 1999). Persons testing positive for the family mutation are advised to have a screening colonoscopy every one to three years from the age of 25 or five years before the age of the youngest cancer occurrence in the family, whichever is earlier (Jarvinen, Aarnio et al. 2000; Renkonen-Sinisalo, Aarnio et al. 2000; Mecklin and Jarvinen 2005). Endometrial screening is currently advised for female mutation carriers although proof of efficacy is lacking (Vasen, Mecklin et al. 1993; Lynch and Lynch

1995; Jarvinen, Aarnio et al. 2000; Dove-Edwin, Boks et al. 2002). Non-carriers of the mutation are advised that their risk of colorectal and other HNPCC related cancers is equivalent to that of the general population from which they come. Mutation carriers found to have synchronous or metachronous cancers in the bowel are often advised to undergo total colectomy with or without rectal sparing. Female mutation carriers may be advised to have prophylactic hysterectomy and oophorectomy at the completion of child bearing (Sumoi, Hakala-Ala-Pietila et al. 1995; Lawes, SenGupta et al. 2002; Church and Simmang 2003; Van Dalen, Church et al. 2003). Screening for other cancers in the HNPCC spectrum such as upper gastrointestinal, ureteric and biliary, has not yet been shown to have an affect on the incidence or mortality of these cancers (Renkonen-Sinisalo, Sipponen et al. 2002). It is reasonable to perform an initial gastroscopy and eradicate H. pylori if present as it is a WHO class 1 carcinogen. In families which seem predisposed to certain of these cancers, screening may be reasonable but evidence is lacking. Examples include urine cytology for ureteric transitional cell carcinoma and abdominal MRI for pancreatic cancer.

Muir-Torre syndrome (MTS) has historically been described as the coincidence of at least one sebaceous skin tumour or keratoacanthoma and one internal malignancy. About half of MTS patients have colorectal cancer. More recently MTS has been shown to be a subtype of HNPCC with germline mutations in genes overlapping with those characteristic of HNPCC seen in about 70% of such patients (Kruse and Ruzicka 2004; Ponti, Ponz de Leon et al. 2005; Ollila, Fitzpatrick et al. 2006). Skin and bowel tumours show MSI (Honchel, Halling et al. 1994; Halling, Honchel et al. 1995).

1.6.3 DNA Mismatch Repair Genes

The DNA mismatch repair (MMR) system spans over a billion years of evolution. It identifies and repairs, in a strand-specific manner, errors such as base/base mismatches and insertion/deletion mutations that result from the activity of DNA polymerase during replication. Runs of base repeats such as polyA tracts or dinucleotide repeats such as (CA)n (microsatellites) are prone to slippage during replication. The MMR system which involves many proteins binds to and excises the mismatched sequence, facilitating the re-synthesis of the correct DNA sequence. In species from bacteria to humans, DNA microsatellite instability due to unrepaired slippage of these mono and dinucleotide tracts, is a feature of genomes or cells that lack MMR activity (Loukola, Eklin et al. 2001). There are nine

mammalian MMR genes comprising *MLH1*, *hMLH3*, *PMS1&2* and *MSH2-6* (Muller and Fishel 2002; Kunkel and Erie 2005). These are the homologs of the bacterial proteins MutL and MutS. They interact with each other to form dimers, trimers and tetramers that mediate distinct functions involved in DNA mismatch repair. Their major function is to identify and repair single base mismatches and insertions or deletions of multiple base pairs which may form redundant 'loops' outside the double DNA strand. The hMSH proteins contact double stranded mammalian DNA scanning along the DNA for mismatches like a 'sliding clamp'. When a base pair mismatch or insertion/deletion of bases is found, repair is initiated. The MSH proteins interact with a number of others including MLH, PMS and EXO1 (Constantin, Dzantiev et al. 2005; Zhang, Yuan et al. 2005). The MSH2-MSH6 heterodimer is thought to primarily repair single base substitutions and 1 bp insertions and deletions while MSH2-MSH3 repairs 1-4bp insertion or deletion mutations (Muller and Fishel 2002). MLH and PMS proteins interact with heterodimers of the MSH proteins to aid in catalyzing their different functions. MLH1-PMS2 is the primary complex that mediates the catalyst function for MSH2-MSH6 heterodimers. Although all nine MMR proteins can be seen to form dimmers in eukaryote cells, not all of these are of known function.
Loss of function within the mismatch repair system allows increasing numbers of mismatch and insertion and deletion mutations to occur, primarily in areas of the genome rich in repetitive DNA sequences. This mutator phenotype may be identified in tissue by instability in alleles with repetitive base sequences, so-called, microsatellite instability.

1.6.4 Germline MMR Gene Mutations

In the early 1990s, almost co-incidentally reports emerged regarding alterations in repetitive base sequences in a subset of colorectal cancer and linkage of Hereditary Non Polyposis Colorectal Cancer to a locus on chromosome two (Peltomaki, Aaltonen et al. 1993) (Ionov, Peinado et al. 1993). Several publications recognised that microsatellite instability was a feature of a subset of human colorectal cancers, often those in persons with a strong family cancer history (HNPCC)(Aaltonen, Peltomaki et al. 1993; Aaltonen, Peltomaki et al. 1994). At the same time Peltomaki et al performed linkage analysis in two large HNPCC kindreds and a putative locus was mapped to chromosome 2p (Peltomaki, Aaltonen et al. 1993). Fishel et al then reported the identification of the human homolog of the bacterial MutS protein, called MSH2 (Fishel, Lescoe et al. 1993; Fishel, Lescoe et al. 1994). This mapped to chromosome 2p22-21 and they suggested that mutations in *MSH2* might be responsible for

HNPCC. MSI had already been studied extensively in bacteria and yeasts and aided the use of positional cloning strategies to identify *MSH2* on chromosome 2p and *MLH1* on chromosome 3p (Fishel, Lescoe et al. 1993; Peltomaki, Aaltonen et al. 1993; Bronner, Baker et al. 1994) .A report was published almost simultaneously by Leach et at inwhichthey reported sequencing the *MSH2* gene and finding mutations in the germline and tumours of affected persons from HNPCC families (Leach, Nicolaides et al. 1993). Meanwhile a second HNPCC locus had been located using linkage analysis at 3p21-23 and tumors in the families involved found to be microsatellites unstable (Lindblom, Tannergard et al. 1993). The gene for the human homolog of MutL was found to be located at this locus and a pathogenic missense mutation found in one family by Bronner et al and almost simultaneously several more pathogenic mutations were identified in kindreds by Papadopoulis et al (Bronner, Baker et al. 1994) (Papadopoulos, Nicolaides et al. 1994). The first reports of PMS2 gerline mutations followed swiftly (Leach, Nicolaides et al. 1993; Peltomaki, Aaltonen et al. 1993; Nicolaides, Papadopoulos et al. 1994). In 1995 the human MSH6 gene was cloned and Miyaki et al discovered several germline mutations in Japanese HNPCC families (Drummond, Li et al. 1995; Miyaki, Konishi et al. 1997). Germline mutations in MLH3 and PMS1 have been postulated but their role in the causation of HNPCC remains uncertain (Lipkin, Wang et al. 2000; Wu, Berends et al. 2001). Tumours from persons in all such families were distinguished by microsatellite instability rather than by regions of allelic loss.

Germline mutations in *MSH2* and *MLH1* are widely distributed throughout both genes. The majorities are point mutations arising from single base-pair substitutions, deletions or insertions. In *MSH2* this results primarily in frameshifts (60%) or premature truncations (23%), whereas frameshifts (40%) or missense mutations, some of which have unknown significance (31%) are observed in the *MLH1* gene (Wijnen, Khan et al. 1996) (Peltomaki and de la Chapelle 1997). Simple assays to detect the functional consequences of these frequent missense mutations are not yet widely available and remain within the domain of research laboratories. Large genomic deletions and duplications in both *hMSH2* and *hMLH1* also occur in the germline in a smaller percentage of HNPCC kindreds Wijnen (Wijnen, van der Klift et al. 1998; Charbonnier, Raux et al. 2000; Wang, Friedl et al. 2003). Alu-repetitive elements appear to be involved in the breakpoints of many deletions which may account for the mutations in up to 10% of HNPCC families (Wijnen, van der Klift et al. 1998). Several specific deletions have been shown to be founder mutations in families from Holland, France and the USA (Charbonnier, Raux et al. 2000; Wagner, Barrows et al. 2003). For example,

three Finnish founder mutations in *MLH1* may account for a slightly higher incidence of the disease in Finland (Aaltonen, Salovaara et al. 1998). The majority of mutations in the MMR genes can be detected with single stranded conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE), denaturing high performance liquid chromatography (DHPLC) and direct sequencing. Detection of large genomic deletions requires other methodology such as Southern bolt analysis, quantitative (real time) PCR and Multiplex Ligand-dependant Probe Amplification (MLPA).

1.6.5 Microsatellite Instability

The loss of MMR gene function leads to a defect in the repair of single base mismatches and of small deletions and insertions in tracts of DNA with repetitive base sequences. Over several generations of cell division, multiple different sized alleles in short repetitive DNA sequences will accumulate in a tumour which is deficient in MMR activity and this is called 'microsatellite instability' (MSI) when it occurs at a microsatellite. Repetitive DNA sequences also occur within genes. Transforming growth factor β receptor type II (TGβRII), BAX, the insulin like growth factor receptor type II (IGFIIR), cell cycle regulated transcription factor E2F-4, TCF4, caspase 5 and even the MMR genes hMSH3 and MSH6 contain such short repetitive sequences and can themselves be affected (Planck, Wenngren et al. 2000; Loukola, Eklin et al. 2001) (Yamashita, Arimura et al. 2000). MSI is seen in about 90 to 95% of HNPCC cancers and is present at widespread microsatellite loci (Frayling 1999). MSI is also present in 10-15% of sporadic colorectal cancers, therefore it is a sensitive but not specific marker for germline mutations of MMR genes (Herman, Umar et al. 1998). Sporadic adenomas very rarely exhibit MSI, therefore the finding of MSI in a dysplastic colorectal adenoma is a sensitive and specific marker for HNPCC (Loukola, Salovaara et al. 1999; Iino, Simms et al. 2000).

There has been and continues to be much debate regarding the definition of a microsatellite unstable cancer. Questions include which microsatellite should be used, how many of them need to be done, what percentage of these need to show MSI and if it matters whether or not a mononucleotide marker is unstable. There is also some disagreement as to how the actual test results should be interpreted for each microsatellite. In 1997 the International Workshop on MSI and RER Phenotypes in Cancer Detection and Familial Predisposition proposed a panel of five microsatellite markers to be used in MSI analysis. This reference panel is

known as the Bethesda markers. It contains two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346 and D17S250) (Boland, Thibodeau et al. 1998). High level microsatellite instability (MSI-H) is said to be present in the tumour if two or more markers are unstable (40% $\leq$ of markers) and low level microsatellite instability (MSI-L) if only one is unstable (40%> of markers). Tumours with no unstable markers are said to be microsatellite stable (MSS). The MSS and MSI-L groups are often combined as a number of studies have shown no phenotypic differences between cancers and individuals with either pattern (Whitehall, Wynter et al. 2002). Also, there is an unknown baseline rate of instability in colorectal cancers such that if enough markers are done almost all will have at least one marker unstable (Halford, Sasieni et al. 2002; Laiho, Launonen et al. 2002) (Tomlinson, Halford et al. 2002). It has been suggested that BAT-26 as a single marker is enough to determine MSI status when the aim is to determine whether an individual carries a germline MMR mutation (Samowitz, Slattery et al. 1999). BAT-26 is extremely reproducible, with a high level of agreement between observers (Cravo, Lage et al. 1999). There are few germline polymorphisms, especially within the Caucasian population (0.7%), hence constitutional DNA is not required for comparison with tumour DNA, unlike all dinucleotide markers. The PCR reaction itself can be done with DNA derived from paraffin preserved archival tissue without the necessity of multiple repeats as is often the case for other markers in the Bethesda group. A study done by Loukola et al in a population of 494 colorectal cancer patients tested for MSI using both BAT 26 and the Bethesda markers showed that 97% of MSI-H cancers were picked up using BAT-26 alone (Loukola, de la Chapelle et al. 1999).

1.6.5 Somatic Mutations in Microsatellite Unstable Tumours

MMR gene defects are presumed to lead to tumour development due to accumulations of widespread mutations in repetitive DNA sequences throughout the genome. Many of these microsatellites lie in non-coding regions and are probably functionally insignificant. Some however are found within exons of genes which may potentially contribute to carcinogenesis. The *type II TGFβ receptor* gene was the first of this kind identified (Lu, Akiyama et al. 1995; Markowitz, Wang et al. 1995; Akiyama, Iwanaga et al. 1996; Percesepe, Kristo et al. 1998). TGFβ is a potent inhibitor of colonic epithelial growth. Frameshift mutations common occurred in a short GT repeat sequence or a polyA tract as a result of insertions/deletions. As mentioned above, other mutational targets include the apoptosis regulator *BAX, IGFR-II,*

E2F-4, TCF-4, caspase 5, CDX2, hMSH3 and *MSH6* (Fujiwara, Stolker et al. 1998; Sakao, Noro et al. 1998; Iino, Simms et al. 2000; Planck, Wenngren et al. 2000; Akiyama, Nagasaki et al. 2001). All of these genes, while frequently mutated in MSI-H tumours and never in MSS tumours, may play a role in the MMR/MSI pathway of tumourigenesis. Other genes with short repeat sequences but unlikely to be involved with tumour formation, the histones for example, do not show instability.

There is still debate regarding the frequency of mutations in *APC, beta-catenin,* K-ras, *TP53, SMAD4* and DCC in MSI-H colon cancers (Table 1.6). Although initial reports suggested that mutations were present in only 21% of MSI-h tumours more recent studies show a high rate of β-catenin mutations in tumours with wild-type *APC*, indicating that over 60% of MSI-H tumours have mutations leading to disruption of the Wnt pathway (Mirabelli-Primdahl, Gryfe et al. 1999; Miyaki, Iijima et al. 1999; Shitoh, Furukawa et al. 2001; Johnson, Volikos et al. 2005), 1999}. Huang et al found a rate of 29 mutations in 52 MSI-H tumours compared with 47 mutations in 63 MSS tumours (Huang, Papadopoulos et al. 1996). Another group has found a very low incidence of biallelic inactivation of *APC* compared to MSS tumours and a total lack of truncating mutations in *APC* in tumours with *TGFβR-II* mutations (Olschwang, Hamelin et al. 1997). It is likely that *BRAF* and K-ras mutations are mutually exclusive in colorectal cancers and that whilst Kras mutations are common in sporadic colorectal cancers and MSI-H cancers from persons with MMR gene mutations, *BRAF* mutations, specifically V600E are more common in sporadic MSI-H cancers (Rajagopalan, Bardelli et al. 2002; Oliveira, Westra et al. 2004; Rowan, Halford et al. 2005).

A study comparing 39 cancers form mutation positive HNPCC patients with 57 sporadic proximal colon cancers (30% of which were MSI-H) found a similar incident of K-ras mutations in codons 12 and 13 in both groups – 30% (Fujiwara, Stolker et al. 1998). G→A in the second nucleic acid position of codon 13 represented 55% of mutations in HNPCC tumours whilst G→A in the second nucleic acid position of codon 12 was over represented in the sporadic tumours. LOH at 18q and p53 gene product overexpression were inversely related to MSI. There was evidence for the mutational spectrum within cancers being dependant on the mechanism for MSI. *BAX* mutations were seen significantly more frequently in HNPCC related MSI-H cancers than sporadic MSI-H cancers and E2F-4 mutations were more common in tumours of patients with germline mutations in *MSH2* (Moriyama, Sasamoto et al. 2002). Mutations in TP53 appear to be less frequent in MSI

cancers than in MSS cancers. The mutation incidence in MSI colon cancers ranges between 0 and 40% in different studies whereas the incidence in MSS cancers is between 31-67% (Konishi, Kikuchi-Yanoshita et al. 1996; Olschwang, Hamelin et al. 1997; Fujiwara, Stolker et al. 1998; Miyaki, Iijima et al. 1999),. The increase in *BAX* mutations may compensate for the low rate of *TP53* mutations in MSI cancers. Some of these differences in these reports are likely to be accounted for by the use of different definitions by various groups of MSI – the number of markers required to be unstable and whether or not mononucleotide markers are included.

Another distinctive finding in MSI-H tumours of both sporadic and familial origin is their tendency to show a diploid chromosome complement as opposed to the majority of sporadic tumours, which show high levels of aneuploidy (Eshleman, Casey et al. 1998; Lindor, Jalal et al. 1998).

1.6.6 Immunohistochemistry for mismatch repair proteins

Mutation or promoter methylation of the MMR genes *MLH1* and *MSH2* is responsible for the majority of MSI-H tumours. Monoclonal antibodies against the protein products of both these genes as well as hMSH6 are commercially available (Kim, Piao et al. 1998). IHC of tumours can help to provide a guide as to whether a tumour has resulted from inactivation of a MMR gene and which gene this is likely to be (Kim, Piao et al. 1998) (Ichikawa, Lemon et al. 1999; Marcus, Madlensky et al. 1999). Lack of MLH1 protein may be due either to germline mutation or to somatic methylation of the promoter (Herman, Umar et al. 1998; Wheeler, Beck et al. 1999). Lack of MSH2 however is almost pathognomonic of germline *MSH2* mutation. MSH2 and MSH6 staining are often lost together in germline *MSH2* carriers as dimerisation of the proteins occurs in vivo. A study which sought to correlate the results of IHC with those of MSI testing in a group of 114 tumours consecutively selected from several different sources found IHC for MMR proteins to be 92.3% sensitive and 100% specific in predicting MSI status (Terdiman, Gum et al. 2001). The observed predictive value of absence of expression of one of the MMR proteins was 100% and for the normal expression of both, 96.7%. Others have shown 100% correspondence between tumour MSI results and tumour IHC (Cawkwell, Gray et al. 1999; Dieumegard, Grandjouan et al. 2000). Several other groups have shown a much lower rate of concordance (Chaves, Cruz et al. 2000; Ward, Meagher et al. 2001). There are several explanations for this. MSH6 mutations

may account for some cases although MSH2 staining is occasionally lost along with MSH6 in tumours from germline *MSH6* mutants. Some missense variants of *MLH1* and *MSH2* may transcribe and translate a stable but non-functional protein, or low levels of stable protein. It is our own experience that decreased levels of staining as well as absence may occur in the tumours of germline mutants. Immunohistochemistry, although relatively cheap and simple involves some skill to perform and report in a reproducible manner and some centres may not have the proficiency of others. MLH1 antibodies in particular may give variable patchy staining (M Novelli, personal communication). On the other hand, IHC testing is more accessible for many service laboratories than MSI testing which involves the use of PCR-based techniques. IHC is now becoming widely used as confirmation of MSI status and to direct mutational testing.

1.6.7 Microsatellite Instability In Pre-Malignant Lesions

HNPCC patients form adenomas at a slightly but not strikingly increased rate compared to the general population. It is widely believed that adenomas in HNPCC patients are more likely to evolve into carcinomas and at a more rapid rate than sporadic colorectal adenomas (Jacoby, Marshall et al. 1995; Vasen, Nagengast et al. 1995; Vasen, Taal et al. 1995). Adenomas in HNPCC patients are more likely to occur at a young age, be large, have a tubulo-villous or villous histology and show severe dysplasia. MSI appears to be an early change in tumours in HNPCC patients, occurring in adenomas fairly frequently (57-80%) and being quite a specific indicator of MMR germline mutation status. A recent study by Kim et al showed that the variability of base numbers in polyA sequences was similar between adenomas and carcinomas in HNPCC, consistent with early loss of MMR activity (Kim, Salovaara et al. 2002). MSI is extremely uncommon in sporadic adenomas (0-3%) and given that 15% of sporadic tumours are MSI-H this points to a later onset of MSI in sporadic tumours. Conversely, the presence of MSI in an adenoma is strong evidence for the presence of a germline MMR mutation (Frayling 1999; Loukola, Salovaara et al. 1999). Evidence also exists for the occurrence of MSI in endometrial hyperplasia in female HNPCC patients (de Leeuw, Dierssen et al. 2000).

1.6.8 Microsatellite unstable sporadic tumours

As previously stated, about 15% of sporadic colorectal cancers and approximately 3% of all sporadic colorectal adenomas show a MSI-H phenotype (Herman, Umar et al. 1998). This has repeatedly been shown to be due to the somatic hypermethylation of the 5' CpG islands in the promoter region of *MLH1* rather than any germline variant (Esteller, Levine et al. 1998; Fleisher, Esteller et al. 2000; Kuismanen, Holmberg et al. 2000; Lind, Thorstensen et al. 2004). Such epigenetic silencing has not been found in any other MMR gene despite the *MSH2* gene also having a CpG island in its promoter region. It leads to a lack of MLH1 protein expression in these tumours on IHC (Young, Simms et al. 2001). Colorectal cancer cell lines with MSI can be shown to have similar hypermethylation which, when reversed, abrogates the MSI present in the cells and establishes re-expression of the MLH1 protein (Brieger, Trojan et al. 2002). Such methylation of *MLH1* may also be a relatively common (46%) 'second-hit' to the wild-type allele for tumours occurring in *MLH1* mutations carriers (Kuismanen, Holmberg et al. 2000). This is controversial with some groups finding almost no methylation in HNPCC tumours and LOH as the major second hit (Wheeler, Loukola et al. 2000). LOH or somatic mutations of *MLH1* are detected in only a small minority of sporadic MSI-H cancers found to have *MLH1* hypermethylation. Methylation of CpG sites upstream of *MLH1* in normal colonic mucosa increases with age and it is postulated that tumour formation starts when a threshold decrease in MLH1 expression occurs. This is in keeping with the known occurrence of MSI-H sporadic cancers in an older population. There is general agreement that MSI is a later phenomenon in the genesis of sporadic MSI-H CRC that in HNPCC related CRC. Hypermethylation of *MLH1* also leads to MSI in gastric cancer, uterine cancer and atypical endometrial hyperplasia (Esteller, Catasus et al. 1999; Fleisher, Esteller et al. 1999; Fleisher, Esteller et al. 2000).

1.6.9 Germline Mutations in *MSH6*

MSH6 (MIM 600678) is one of the MMR genes less commonly mutated in the germline and the associated disease has some distinctive features in both molecular pathology and phenotype of affected families. The first family with a germline MSH6 mutation was described in the literature in 1997 (Miyaki, Konishi et al. 1997). The family did not meet the Amsterdam criteria for HNPCC. There was a high incidence of both endometrial and ovarian cancer and a relatively late onset of tumours with a colon cancer from one member being MSI-H. In another study, 25 germline MSH6 mutations were found in a group of 316 probands of HNPCC-like families (Berends, Wu et al. 2002), Twelve had truncating

mutations and a further 13 had missense mutations though to be pathogenic. 54% of endometrial and colorectal tumours from mutation carriers were MSS or MSI-L. 66% of tumours from carriers of truncating mutations and 18% with missense changes lacked MSH6 protein immunostaining. Numbers of patients affected and their age at cancer diagnosis did not differ between those with truncating and missense mutations.

There are some common findings regarding *MSH6* mutation carriers. Disease occurs at a later age on average and there appears to be a lower penetrance compared to MLH1/MSH2 carriers. This is possibly due to the loss of MSH6 function causing only a partial MMR defect. There is risk of endometrial cancer development among female carriers of around 70%, and colorectal tumours appear to have a left sided predominance (Wijnen, de Leeuw et al. 1999) (Berends, Wu et al. 2002),. Although the majority of cancers in persons with germline *MSH6* mutations will show loss of *MSH6* expression and high frequency MSI, some will not. Thus proceeding to germline *MSH6* mutation testing may be reasonable in some HNPCC-like families with CRC and endometrial cancer and whose tumours are not MSI-H (Wagner, Hendriks et al. 2001; Hendriks, Wagner et al. 2004).

1.6.10 *EXO1*

The yeast homologue of *EXO1* has been shown to interact with *MutS* and strains deficient in EXO1 have mutator phenotypes and dinucleotide repeat instability. The human protein product EXO1 also interacts with MSH2 and is involved in the MMR process. In a first report, this gene was screened as a candidate in 33 probands from Amsterdam positive HNPCC families and in 225 probands from HNPCC-like families (Bethesda criteria) (Wu, Berends et al. 2001). Germline *EXO1* variants were found in 14 patients, one splice site and 13 missense mutations. None of the 13 missense variants were present in 200 controls. Five missense variants were found in more than one patient. Seven of the fourteen patients had tumours which were MSS. In tumours of those with germline variants, 12 had loss of the variant allele and retention of the wild-type one.

A further study cast some doubt on the assertion that germline *EXO1* mutations have a pathogenic role. Jagmohan-Changur et al evaluated a series of 970 CRC patients and 1007 controls from a number of countries for the EXO1 variants found in the previous study plus any novel variants (Jagmohan-Changur, Poikonen et al. 2003). In an initial group of 20

controls in which EXO1 was fully sequenced, all but one of the previously described EXO1 variants was present. The Pro640Ser variant still remained as a possible pathological mutation. Five of nine previously described mutations including the truncating variant IVS12-1 (G→C) were detected in patients and controls with equal frequency. None of the missense variants occurred in a conserved amino acid. Functional studies have not been performed on any of the missense variants. They concluded that is was unlikely that germline variants in EXO1 of themselves can cause an HNPCC phenotype in a family. It is still possible that EXO1 has a role as a low penetrance allele causing predisposition to colorectal cancer.

1.6.11 Population-based Diagnosis of HNPCC

The lifetime risk of colorectal cancer in Western populations is approximately 1 in 20 to 25. Rates are lower but rapidly increasing in Asian and oriental societies. Causes of colorectal cancer include lifestyle factors including exercise, weight, diet, especially red meat intake and smoking as well as inherited genetic factors. For largely unknown reasons, a family history of colorectal cancer is a major risk factor and is present in 15 to 20% of those affected. Risk increases with decreasing age and increasing number of relatives affected. Germline mutations in the MMR genes are responsible for a small but significant proportion of colorectal cancer incidence, 2-5% in most populations. The number of kindreds who may potentially carry deleterious mutations may be underestimated given the limitations of modern small family size, early deaths due to non-cancer related causes and uncertain ascertainment of disease in prior generations. Therefore much effort has been directed towards the use of tumour samples from kindreds suspicious for HNPCC for MSI testing and immunostaining as predictors of germline mutation status. These tests are relatively simple and cheap compared with germline mutation analysis and can pin-point the gene involved, making mutation analysis of only one gene in germline DNA necessary.

Using clinical criteria alone for deciding upon mutation testing will exclude a significant proportion of families. Using the Amsterdam criteria alone a high number of HNPCC families will be missed. Winjen et al examined a series of 125 colorectal cancer families for germline MMR mutations. 49% of those meeting the Amsterdam criteria had a detectable pathological germline mutation compared with 8% of those who did not totally meet the criteria (Wijnen, Vasen et al. 1998). The original Amsterdam criteria has a sensitivity of

about 60% with a specificity of 70%. Using the modified Amsterdam criteria (table 1) sensitivity rises almost 80% with a specificity of 62%. If all patients and families who meet Bethesda criteria are tested, sensitivity rises to 95% but specificity falls to 25% (Syngal, Fox et al. 2000). It would involve huge resources to perform germline mutation resting on all families included in the Bethesda criteria, but it does not seem unreasonable to use MSI testing and/or immunostaining for MMR proteins to narrow this group down to those who will go on to germline mutation testing. In practice this is increasingly being done (Ichikawa, Lemon et al. 1999; Vasen, Hendriks et al. 2004; Stormorken, Bowitz-Lothe et al. 2005).

In 1998, Aaltonen et al produced criteria for MSI testing of tumours (Aaltonen, Salovaara et al. 1998). After testing a series of 509 consecutive patients' cancers for MSI and loss of MLH1 and MSH2 immunostaining and then performing mutation analysis in those with MSI-H tumours (and finding 10 germline mutations), it was felt that the same germline mutations would have been discovered if only a subset of tumours had been tested. If only all patients affected under 50 years, all patients with first degree relatives with colorectal or endometrial cancer and all patients with multiple primary cancers in the colorectum, endometrium or both had been tested for MSI, no patient found to have a germline mutation would have been missed. This would have involved testing 24% of patients presenting with colorectal cancer.

Aaltonen's criteria were prospectively tested in a group of 535 CRC patients (Salovaara, Loukola et al. 2000). BAT-26 was used to determine MSI status in all cancers. Those who were MSI high went on to have mutation testing done. Had the above criteria been applied (patients affected under 50 years, all patients with first degree relatives with colorectal or endometrial cancer and all patients with multiple primary cancers in the colorectum, endometrium or both), 117 (22%) of patients would have undergone MSI testing and 17 of these would have been diagnosed with germline mutations. This would have missed one germline mutation in this population. All patients tested in both studies would also have been identified by the application of the Bethesda criteria, both original and revised.

In a similar prospective study, 257 unselected United State patients with CRC whose tumours were tested for MSI and loss of MLH1, MSH2, MSH6 protein products (Cunningham, Kim et al. 2001) . Fifty-one (20%) tumours were MSI high. Of these 48 lacked MLH1 protein staining and 3 lacked both MSH2 and MSH6 immunostaining. All of these patients were examined for HNPCC mutations. Four *MLH1* germline mutations were detected and three

MSH2 mutations. Hypermethylation of the *MLH1* gene promoter was seen in all cancers with absent MLH1 expression but no germline mutation. Overall 1.9% of the cohort tested had a germline mutation in a MMR gene.

A large study also in the United States by Samowitz et al examined 1066 CRC patients obtained retrospectively through Kaiser Permanente medical Care Program (Samowitz, Curtin et al. 2001). Using BAT-26 and TGFβRII microsatellite markers, 14% (131/933) of tumours were classified as MSI-H. Germline sequencing was performed on 130 of these individuals revealing seven probable HNPCC mutations. Two of these seven had neither a family history of HNPCC-related cancer nor young age at diagnosis although the methods used to ascertain family history were not mentioned in the study. This correlated to an estimated 0.86% of cases of colorectal cancer occurring in HNPCC mutation carriers. A further 12 probands had missense changes of uncertain significance at least six of which were unlikely to be pathogenic.

In another recent study performed in Italy, Percesepe et al prospectively collected 336 cases of CRC and tested them for MSI Percesepe (Percesepe, Borghi et al. 2001) . MSI-H cases were then stained for loss of MMR protein and *MLH1* methylation studies were performed. 8.3% (12) of the cancers showed high level MSI. Of this group, only one was shown to carry a germline mutation in MSH2 to give a population incidence of 0.3%. Seventy-one percent (20) of MSI-H carcinomas showed lack of expression of MLH1 of which 14 had MLH1 promoter methylation. No tumours showed loss of MSH2 staining. However in this entire cohort, apparently only 6 patients met the Amsterdam criteria (3 MSI-H and 3 MSS) and these were apparently, the only ones tested for germline MMR mutations.

Farrington et al studied a specific population of CRC probands diagnosed under age 30 (Farrington, Lin-Goerke et al. 1998). Twenty eight percent were found to have pathologic MMR mutations. In this cohort the sensitivity and specificity for HNPCC of having a MSI-H tumour were 86% and 73% respectively with positive and negative predictive values of 63% and 90%. Interestingly, in this study only 21% of patients with germline mutations came from patients whose family met Amsterdam criteria. It is possible that some represented a new mutation event within the family.

Other studies have shown that immunostaining alone is 90 to 95% sensitive for germline mutation status (Chaves, Cruz et al. 2000) (Dieumegard, Grandjouan et al. 2000; Terdiman, Gum et al. 2001). The specificity of MLH1 immunohistochemistry is low in the setting of general population screening as it is present in about 15% of sporadic MSI-H cancers with epigenetic silencing of *MLH1*.

Thus there exist a number of different models for screening populations for HNPCC. In populations where common founder mutations do not exist, mass mutation analysis is impractical. Genetic 'pre-testing' with MSI and immunohistochemical tests appears to be the most practical alternative. All groups agree on a Bethesda-like approach to who should be tested with IHC and MSI. Those diagnosed under age 50, those with multiple tumours, and those with significant family history especially those including endometrial and CRC are agreed upon. It seems practical to perform IHC at the time of cancer diagnosis for such persons. Lack of MLH1 immunostaining may imply either MLH1 somatic methylation or germline MLH1 mutation. The probability of the latter depends largely on pretest probabilities. The younger the affected person and the stronger the family history, the higher the chance of a mutation being found. If doubt exists and a second tumour is available within the family, testing it with IHC or MSI testing can clarify the issue. This is particularly helpful when no live affected family members exist and such testing is being done as an aid to screening recommendations for family members.

1.7 Colorectal Cancer Screening

1.7.1 Colorectal Cancer Screening For Persons At Average Risk

The benefits of any screening program depend on several factors; the incidence of the condition in the population being screened, the existence of a premalignant lesion or early lesion which can be detected and effectively treated, the sensitivity and specificity of the test, the morbidity and acceptability of the screening procedure. To a greater or lesser extent in any screening program, health economics will dictate how expensive the screening procedure and confirmative tests will be and what the costs per year of life saved by screening will be. Colorectal cancer presents one of the most compelling arguments for population screening strategies. It is common, a non-malignant precursor exists and colonoscopy is highly sensitive and specific and allows removal of the pre-malignant lesion at the time of the

procedure. Severe adverse complications are low and, at a population level, five to ten yearly or even once-off colonoscopy does not present a huge financial burden. Faecal occult blood testing (FOBT) leads to a 30% reduction in deaths from colorectal cancer (Mandel, Church et al. 1999; Ransohoff and Pignone 2001). It has a fairly high sensitivity for adenomas of significant malignant potential however false positive tests are fairly common. There are four major tools in use at the moment for colorectal cancer screening; FOBT, sigmoidoscopy, double contrast barium enema and colonoscopy. Many guidelines for the screening of average risk populations have been published but as yet no strategy exists on a national level in the UK (Rhodes 2000; Smith, Mettlin et al. 2000; Pignone and Levin 2002; Rex 2002; Smith, Cokkinides et al. 2002; Winawer, Fletcher et al. 2003; Pignone 2004; Winawer, Faivre et al. 2005; Winawer 2005). There have been three major trials using guaiac-based FOBT as a screening measure to attempt to reduce mortality from CRC, from Minnesota USA, Denmark and the United Kingdom (Mandel, Bond et al. 1993; Hardcastle, Chamberlain et al. 1996; Kronborg, Fenger et al. 1996; Mandel, Church et al. 1999). The Minnesota trial compared annual and biennial testing with no screening in a prospectively randomised group of 46,551 persons. Most FOBT test cards were rehydrated which increases sensitivity from 40% to 50-60% whilst reducing specificity from 96-98% to 90%, giving more false positive tests. (Church, Ederer et al. 1997; Lang and Ransohoff 1997; Ransohoff, Lang et al. 1997). Cumulative cancer mortality rates after 18 years of follow-up were 33% (CI, 17-49%) lower among persons randomised to annual FOBT than those randomised to no screening. Biennial screening, which did not show a reduction in mortality at 13 years follow-up, produced a 21% (CI, 3-38%) reduction in mortality rate at 18 years. The 18 year follow-up also showed a 20% (CI, 10-30%) decrease in the incidence of CRC in the group screened annually compared to controls. The more recent trials from the United Kingdom and Denmark by Hardcastle et al and Kronborg et al used biennial testing only with guaiac-based testing cards and no rehydration of cards (Hardcastle, Chamberlain et al. 1996; Kronborg, Fenger et al. 1996). Reductions in CRC mortality of 15% (CI, 1-26%) and 18% (CI, 1-32%) were found in these two trials respectively. In the Minnesota trial, 28% of participants required colonoscopy for positive tests whilst in the two European trials, only 5% did so (Mandel, Bond et al. 1993; Hardcastle, Chamberlain et al. 1996; Kronborg, Fenger et al. 1996). This may be due to the increase in sensitivity with reduced specificity seen with FOBT card rehydration. In these three studies overall, FOBT detected 27-39% of patients who developed cancer in the screening group. This is an impressive results and the test itself is easy and cheap to perform and has no adverse effects. Nonetheless, there is a frequent

requirement for colonoscopy, which can occasionally have adverse effects. The sensitivity of FOBT for detection of adenomas of low to moderate malignant potential is fairly low (<50%) and false reassurance may result from a negative test. It is noteworthy that semi-quantative FOBT based on immunochemistry can detect as little as 100ng of haemoglobin per ml of faeces. The tests can be 'set' to read positive at a given level of haemoglobin. Thus sensitivity can be adjusted up or down depending on the number of screening colonoscopies deemed acceptable within a screening program (Fraser, Matthew et al. 2006). There is agreement that whilst FOBT may be a suitable screening strategy for low risk populations it has little place as a sole modality in moderate to high risk ones.

Flexible sigmoidoscopy is currently being employed in two randomised trials, one in the UK using once only flexible sigmoidoscopy and one in the USA of five yearly sigmoidoscopy in conjunction with FOBT (Gohagan, Prorok et al. 2000; Atkin, Edwards et al. 2001; Atkin, Rogers et al. 2004). Two case-control studies one using rigid and one using flexible sigmoidoscopy have been published. Selby el al used data from the Kaiser Permanente Medical Care Programme (Selby, Friedman et al. 1992). They found that rigid sigmoidoscopy had been performed in 9% of persons who died of CRC occurring within 20cm of the anus and in 24% of persons who did not die of such a cancer. The adjusted odds ration of 0.41 (CI, 0.25 to 0.69) suggested that sigmoidoscopy screening reduced the risk for death by 59% for cancer within reach of the rigid sigmoidoscope. The risk reduction continued for 9 to 10 years after the test was performed. Thiis-Evensen et al performed a small randomized trial of flexible sigmoidoscopy in 800 persons (Thiis-Evensen, Hoff et al. 2001). The relative risk reduction for diagnosis of colorectal cancer for screened persons was 0.2 (CI, 0.03-0.95) and for mortality was 1.57 (CI, 1.03-2.40) largely due to cardiovascular deaths. Sigmoidoscopy has a sensitivity for diagnosis of left sided colorectal cancers and polyps of around 70 to 80% with a false negative rate that is operator - dependent (Imperiale, Wagner et al. 2000; Lieberman, Weiss et al. 2000; Atkin, Rogers et al. 2004). Adverse effects are rare, bleeding in 3%, severe pain in 0.4% and bowel perforation in only two in 49,501 sigmoidoscopies were reported in a recent study (Anderson, Pasha et al. 2000). Advantages of sigmoidoscopy include the lack of lengthy and uncomfortable bowel preparation and anesthesia and the ability of the operator to remove polyps during the procedure. The major drawback of such an approach is the failure to visualise the proximal portion of the bowel. The accuracy of sigmoidoscopy can be improved somewhat by the addition of FOBT (Berry, Clarke et al. 1997; Rasmussen, Kronborg et al. 1999).

Double contrast barium enema has not been studied in randomised or case control studies as a screening procedure. Its sensitivity is 32% for polyps smaller than 0.4 cm, 53% for polyps 0.6 to 1.0 cm and 48% for polyps larger than 1cm (Winawer, Stewart et al. 2000). Adverse events are very rare. Discomfort is comparable to colonoscopy as is the length of the procedure.

The ability of colonoscopy to prevent CRC and mortality from CRC has not been prospectively measured in a screening trial in a population at average risk. Information from the National Polyp Study allows an estimation that 76% to 90% of CRC could be prevented by regular surveillance colonoscopy, based on comparisons with historic controls (Winawer, Zauber et al. 1993). However, all trial participants had polyps detected and removed at the time of entrance, limiting the applicability of this study to an average population. A case-control study found that patients with CRC were less likely to have had a previous colonoscopy. Odds ratios for disease incidence were 0.47 (CI, 0.37-0.58) for colon cancer and 0.61 (CI, 0.48-0.77) for rectal cancer. Colonoscopy is highly sensitive for large adenomas and cancers (>90%) and about 75% for smaller adenomas. This is operator dependant to some extent. Serious adverse events will occur in 0.3% of patients, most often bleeding or perforation, especially after polypectomy (Anderson, Pasha et al. 2000). It must be remembered that over a lifetime of regular screening, if the pre-test probability of cancer is small, the number of people harmed by screening could outway the number who benefit from it.

As the plethora of published guidelines and position statements attests, there is little consensus on the need for, method and timing of colorectal cancer screening in the average risk population. Many suggest starting screening at the age of 50 to 55 and recommend five yearly sigmoidoscopy or ten yearly colonoscopy with yearly FOBT.

1.7.2 Colorectal Cancer Screening For Persons At Above Average Risk

More (but only slightly more) agreement can be found in the arena of screening for moderate and high risk persons. Because in the familial syndromes described above, incidence is so high, false negative tests may be fatal, especially for HNPCC mutation carriers who may have rapid progression from adenoma to carcinoma; colonoscopy is the

method of choice for screening the large bowel. In HNPCC the lifetime risk of colorectal cancer is 70 – 80%. A recently completed controlled 15 year trial of 3 yearly colonoscopic screening vs no screening in two cohorts of patients from HNPCC families showed a reduction in colorectal cancer incidence of 62% (Jarvinen, Aarnio et al. 2000). In mutation positive subjects there was over 50% decrease CRC incidence in screened persons. None of the cancers detected in the screened group led to death compared with 9 in the control group. Overall, the death rate in mutation positive subjects was reduced by 66% in the screen cohort ($p=0.05$). The current recommendation for HNPCC mutation carriers is for yearly screening with colonoscopy starting at 25 years of age or five years before than the earliest age of diagnosis in the family, whichever is the younger age (Dunlop 2002; Halbert, Lynch et al. 2004). Prophylactic colectomy and ileorectal anastomosis are often considered in mutation carriers who present with a colon cancer. There is no randomised or controlled evidence for benefit of this practice at present. The risk of cancer in the retained rectum is 3% every three years for the first 12 years so endoscopic surveillance of the rectum is necessary after colectomy. Screening for uterine cancer in female family member is widely performed but has unproven effectiveness (Dove-Edwin, Boks et al. 2002). Studies using newer methods of screening such as hysteroscopy with biopsy may show greater sensitivity and specificity. Screening for other HNPCC cancers such as stomach and transitional cell cancer of the urinary system is performed in some families in which these cancers occur. There is no proof for efficacy at present (Renkonen-Sinisalo, Sipponen et al. 2002).

The same frequency of colonoscopic screening may be necessary for families meeting the Amsterdam criteria but without MSI-H cancers or known HNPCC mutation. In a recent study by Lindor et al it has been suggested that colorectal cancer incidence is significantly lower and that there is no excess risk of extra-colonic cancers in families which meet Amsterdam criteria but whose tumours do not have a MMR-deficient phenotype (Lindor, Rabe et al. 2005). Screening for families with more moderate degrees of risk is somewhat empirical although there have recently been attempts to standardise this in Great Britain and in Australia (Dunlop 2002). For persons with two or more first degree relatives affected with CRC, not meeting the modified Amsterdam criteria, five yearly colonoscopy beginning at age 45 or ten years or earlier than the youngest cancer in the family (or a variant of this) is the normal recommendation. Other pedigrees such as two affected parents, one affected FDR< 40 or 45 and one first and one or more second degree relatives attract less overall agreement regarding screening. Microsatellite testing and immunostaining of tumours for

loss of MMR proteins can be a helpful adjunct to deciding on screening intervals in some families, especially if germline mutation analysis is not possible.

Screening for patients with FAP is best done in specialist centres with expertise in the genetic and clinical aspects of the disease. In the majority of FAP families an *APC* or *MYH* mutation can be detected allowing non-carriers to avoid screening. At-risk individuals from families with classic FAP without known mutation require annual flexible sigmoidoscopy from the early teens until age 30 and then at three to five year intervals until age 60 if no polyps develop. Affected persons require colectomy in their teens to early twenties. Options include total colectomy with ileorectal anastamosis and total proctocolectomy with ileostomy or with ileoanal pouch formation. This risk of cancer in the retained rectum is 12-29% and the rectum must be examined yearly. Polyps can also form in the ileal pouch which must also be kept under review (van Duijvendijk, Vasen et al. 1999). FAP patients also require upper gastrointestinal surveillance although the efficacy of this approach is not supported by a high level of evidence. Two to three yearly oesophagogastroduodenoscopy (OGD) with a side viewing endoscope is used although this is increased to yearly in the case of Spigelman stage III to IV duodenal polyps. These polyps are extremely difficult to treat and still result in a mortality of five to 10% in FAP patients after colectomy (Bulow, Alm et al. 1995; Bulow, Bjork et al. 2004). It is highly important that registries be established to record the results of all screening colonoscopies in these familial conditions so that better prospective estimates can be made of frequency and types of polyps and tumours occurring in these populations. Interval cancers, prophylactic surgery and outcome of cancer treatment should be a part of this record.

1.8 Inflammatory bowel disease

Inflammatory conditions of the colorectum can also lead to an increased risk of carcinoma. Ulcerative colitis and Crohn's disease are the two most common conditions in this category. This risk increases with duration of disease, early age of onset of disease and pancolitis. Ulcerative colitis associated cancers show several distinctive features when compared to sporadic colorectal cancer (Colliver, Crawford et al. 2006). They are predominantly left sided and often mucinous. They are frequently multiple, arising from flat rather than polypoid adenomas, possible because the sequence of dysplasia in the epithelium of the bowel is in the nature of a 'field defect' (Usaj, Tarabar et al. 2004). Precursor areas of flat

dysplasia appear to be just as frequent on the right side of the colon, however, hinting at additional factors in the transition from dysplasia to neoplasia (Rutter, Saunders et al. 2004). Somatic *APC* mutations appear to be less common than in sporadic cancers and it has been suggested that TP53 mutations occur as a much earlier event, possibly as a means of escape from apoptotic stress due to continuing inflammation (Matsumoto, Yoshida et al. 2003; Rosman-Urbach, Niv et al. 2004). Although there is clearly an inherited component in both Crohn's disease and UC, the genetics is complicated and probably confounded by environmental factors, which interact to mediate the immune and non-immune responses controlling inflammation. Many chromosomal regions have been described which contain putative susceptibility loci, some of which have been confirmed IBD1 (16P12-Q13), IBD2 (12p13.2-a24.1) IBD3 (the major histocompatibility complex on chromosome 6) and IBD4 (14A11-12)) with others that await confirmation ; 1p36, 3q, 4q, 5q, 7q, 14q and 19p (Cho 2001; Lawrance, Fiocchi et al. 2001).

1.9 Familial Colorectal Cancer

Colorectal cancer is a common disease with a lifetime risk of developing of disease 1:20 to 1:25 in western societies and a 1:50 lifetime risk of death. Thus it can appear to cluster in families by chance. Despite this, more familial clustering occurs than could be expected by chance and not all such families will fit the modified Amsterdam criteria. Much of the 10 to 20% of colorectal cancer due to inherited factors is still unexplained. Even of families fulfilling the Amsterdam criteria, just over one half will have identified mutations in the MMR genes and up to a third will not display a MSI-H phenotype in their tumours. Good epidemiological evidence exists for the presence of other high and possible low penetrance predisposition factors. Winawer showed that in patients participating in the National Polyp Study the relative risk of colorectal cancer was 1.78 (95% CI 1.18-2.67) in parents and siblings of persons found to have a polyp and that this increased to 2.59 (95% CI 1.46-4.48) when the polyp was diagnosed at age <60 years (Winawer, Zauber et al. 1996). If one first degree relative had a polyp and another a colorectal cancer, RR was 3.25 (95% CI 1.92-5.52). Fuchs et al found that in a very large cohort of men and women with data on family history of colorectal cancer and who were prospectively followed up for the occurrence of CRC that the RR for men with at least one FDR was 1.64 (95% CI 1.04-2.58 and for women was 1.77 (95% CI 1.32-2.37) (Fuchs, Giovannucci et al. 1994). This RR was markedly higher for members of the cohort with a FDR affected under 45 years (5.37) and approached

one for those with one FDR affected at over 65 years of age. For persons with two or more affected FDR, RR was 2.75 (95% CI 1.34 to 5.63). A case control study performed with over 2,500 unselected persons with colon cancer gave similar results for odds ratios (OR) for developing cancer (Slattery and Kerber 1994). Men with one FDR affected by colon cancer had an OR of 2.1 (95% CI 1.65 – 2.69) and women had an OR of 2.57 (95% CI 1.99 – 3.33). These ORs went up considerably if two or more relatives were affected or one relative was affected at under 45 yeas however there were only small numbers in these analyses. Houlston et al found similar degrees of risk amongst their cohort with a lifetime risk of colorectal cancer of 1:6 for those with two affected FRD's and 1:10 for those with one FDR affected under 45 years (Houlston, Murday et al. 1990). Some of these families were recruited through St Mark's Hospital.

This work has been, first and foremost, aimed at explaining inherited aspects of colorectal cancer and its precursor the adenoma. The starting point has been the 2300 families comprising the Bobby Moore database at St Mark's Hospital Family Cancer Clinic. These families have been seen at the clinic for advice and counseling on their family risk and need for colorectal cancer screening. Some families have undertaken discussions regarding specific gene testing and proceeded with such testing. Many individuals from these families have screening colonoscopies performed at St Mark's Hospital. Other families have been recruited through the Clinical Genetics Unit at Guy's Hospital. They are, as a group, very enthusiastic about research into the possible inherited causes of colorectal cancer.

When this work has initiated there were many families on the database who appeared to have an hereditary predisposition to colorectal cancer but in whom known genetic conditions had not been excluded. My first task was to perform microsatellite instability testing and immunohistochemistry on tumours from affected members of these families and then, where possible, to perform mutation analysis on germline DNA to uncover those families with true HNPCC. Whilst doing this it became apparent that there were a number of families who may well have harboured MMR gene mutations but in which there were no live affected members available for genetic testing. I set about developing an algorithm using all the clinical data from the family as well as pathological information and molecular data from the tumours to divide families into those which were truly HNPCC and likely to be mutation positive and those unlikely to have a mutation detected. Such an algorithm can help decide on screening

for families who cannot undergo genetic testing and who may not fulfill the Amsterdam criteria.

Once the mutation positive HNPCC families had been fully described, the next task was to try to group the non-HNPCC families into groups for further studies. The clinical features of individuals and families and the molecular features of their tumours were analysed thoroughly before using a cluster analysis technique to partition the families into strata for further analysis.

One group of families appeared separate from the outset. These were families with multiple colorectal polyps and colorectal cancer. Many of these families had been examined for germline *APC* variants previously and were examined again as part of this study. A significant number did not have detectable *APC* mutations – both families with autosomal dominant and recessive appearing pedigrees. A minority was found to have the CRAC1 haplotype. We have used several approaches in the course of this work to elucidate the genetic basis of this patient group characterized by the presence of multiple colonic adenomas.

Initially a candidate gene approach was used and germline DNA in multiple adenoma families was screened for pathogenic variants in genes involved in pathways known to be important in colorectal carcinogenesis. These comprise a set of genes, which on the basis of their product's activity should play a vital role in one of the known pathways disrupted in carcinogenesis. Many of these genes can be deduced from work in lower species and their human homologue then identified on the basis of sequence similarity. As new pathways involved in malignant change within cell emerge and increasing numbers of human genes are cloned, this list of genes changes and increases. Target genes for this kind of screening can also be deduced from those frequently found to be mutated in sporadic cancers.

Secondly linkage studies can be performed based on the assumption that high or moderate penetrance genes remain to be identified such as the CRAC1 locus on 15q. Large non-parametric linkage studies are currently also being performed with the additional aim of uncovering low penetrance alleles contributing to the familial clustering of colorectal cancer. Such studies are particularly difficult to perform as large numbers are needed, requiring blood collection on a huge scale. Ascertainment of affection status can be problematic when

it relies on self-report from family members, and mutation of known germline predispositions must be excluded in each family.

During the course of the work we were able to verify that a new colorectal adenoma and cancer predisposition gene, *MYH*, does account for a number of these cancers and I was able to go on to describe the pathway of tumourigenesis in such tumours.

Table 1.1 ICG Characteristics of HNPCC

1.	Familial clustering of colorectal cancer and/or endometrial cancer
2.	Associated cancers; cancer of the stomach, ovray, ureter/renal pelvis, brain, small bowel, hepatobiliary tract and skin (sebaceous tumours)
3.	Development of cancer at an early age
4.	Development of multiple cancers
5.	Features of colorectal cancer: (i) predilection for proximal colon (ii) improved survival (iii) multiple colorectal cancer (iv) increased proportion of mucinous tumours, poorly differentiated tumours, and tumours with marked host-lymphocytic infiltration and lymphoid aggregation at the tumour margin
6.	Features of colorectal adenoma: (i) the numbers vary from one to a few (ii) increased proportion of adenomas with a villous growth pattern and (iii) a high degree of dysplasia (iv) probably rapid progression from adenoma to carcinoma
7.	High frequency of microsatellite instability
8.	Immunohistochemistry: loss of MLH1, MSH2 or MSH6 protein expression
9.	Germline mutation in MMR genes (MSH2, MLH1, HSH6, PMS1, PMS2)

Table 1.2 Classic ICG-HNPP Criteria (Amsterdam Criteria I)

- There should be at least three relatives with CRC; all the following criteria should be present:-
- One should be a first degree relative of the other two.
- At least two successive generations should be affected.
- At least one CRC should be diagnosed before age 50.
- Familial Adenomatous Polyposis should be excluded.
- Tumours should be verified by pathological examination.

Table 1.3 Revised ICG-HNPCC Criteria (Amsterdam criteria II)

There should be at least three relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter or renal pelvis)

One should be a first degree relative of the other two.
At least two successive generations should be affected.
At least one should be diagnosed before age 50.
Familial Adenomatous Polyposis should be excluded in the CRC case(s) if any.
Tumours should be verified by pathological examination.

Table 1.4 Bethesda Guidelines for MSI testing of tumour.

1.	Individuals with cancer in families that meet the Amsterdam Critera
2.	Individuals with two HNPCC-associated cancers, including synchronous and metachronous CRC or associated extra-colonic cancers*
3.	Individuals with CRC and a first degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma diagnosed at age<40 years.
4.	Individuals with colorectal cancer or endometrial cancer diagnosed at age <45 years.
5.	Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age <45 years.
6.	Individuals with signet-ring-cell-type CRC diagnosed at aged <45.
7.	Individuals with adenomas diagnosed at age <40 years.

Table 1.5 Revised Bethesda Guidelines for MSI testing of tumour.

Tumours from individuals should be tested for MSI in the following situations
1. Colorectal cancer diagnosed in an individual who is less than 50 years of age. 2. Presence of synchronous, metachronous colorectal, or other HNPCC associated tumours*, regardless of age. 3. Colorectal cancer with MSI histology^, diagnosed in a patient who is less than 60 years of age. 4. Colorectal cancer diagnosed in one or more first degree relatives with an HNPCC related tumour, with one of the cancers being diagnosed under age 50 years. 5. Colorectal cancer diagnosed in two or more first- or second degree relatives with HNPCC-related tumours regardless of age. • *HNPCC-related tumours include colorecta, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract and brain (usually glioblastoma as seen in Turcot syndrome) tumours, sebaceous gland adenomas and keratoacanthomas in Muir-Torre Syndrome and carcinoma of the small bowel. • ^Presence of tumour infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet ring differentiation or medullary growth pattern.

Table 1.6 Molecular genetic features of MSI-H tumours.

	APC (%)	B-Cat	P53 (%)	K-ras (%)	SMAD4 or 18q LOH (%)	BAX	TGF RBII
Miyaki (Miyaki, Iijima et al. 1999)	6/28 (21)	12/28 (43)					
Sakao (Sakao, Noro et al. 1998)						11/19 (58)	
Konishi (Konishi, Kikuchi-Yanoshita et al. 1996)	3/19 (16)		4/20 (20)	1/120 (5)	1/7 (14)		13/20 (65)
Fujiwara (Fujiwara, Stolker et al. 1998)			2/55* (3.6)	14/25 (27)	0/11 (0)	19/46 (41)	46/54 (81)
Huang (Huang, Papadopoulos et al. 1996)	28/52 (54)						
Olschwang (Olschwang, Hamelin et al. 1997)	1/9 (11)		4/13 (31)	2/9 (22)			13/13 (100)

*P53 immunostaining only. Gene product overexpression in >50% nuclei.

1.10 References

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Chapter 2 Materials and Methods

2.1 DNA Extraction

2.1.1 DNA Extraction from Blood

DNA extraction from fresh or frozen blood was performed using either a DNA extraction kit (Nucleon), according to the manufacturer's instructions, or using the ammonium acetate method set out as follows. The first steps of this method acted to break down the cell wall to allow access to the nucleus. Nine ml frozen blood samples were thawed and the blood transferred to a 50ml conical bottom Falcon tube (Greiner). Ice cold water was added to the tubes to give a final volume of 50mls, and then the tube inverted to mix and lyse the red blood cells. The tubes were then centrifuged at 2300rpm for 25 minutes at 4°C in a swing out rotor centrifuge (CR412 Jouan). The supernatant was discarded by inverting the tube gently, being careful not to disturb the pellet. The tube was inverted and placed on a clean paper towel to remove the last traces of supernatant. The pellet was then washed with 25 ml 0.1% NP-40 (Sigma) and centrifuged at 2300rpm for 20 minutes at 4°C. The wash was repeated if necessary. The supernatant was discarded and the tube inverted over a paper towel. To lyse the nuclei, 3ml nuclei lysis buffer was added to the pellets and the tube vortexed to re-suspend the pellet completely. 200µl 10% SDS and 600µl proteinase K solution was then added to the tube to degrade any protein. The solutions were mixed by inversion and incubated at 60°C for 11/2-2 hours or overnight at 37°C. Following proteinase K digestion, 1ml of saturated ammonium acetate solution (148g NH_4Ac (BDH) in 50mls distilled water) was added and the tube vortexed vigorously for 15 seconds. The tube was left to stand at room temperature for 20 minutes, and then centrifuged at 2300rpm for 20mins at room temperature. The DNA was then in the supernatant and ready to be precipitated. The supernatant was transferred to a clean tube 50-ml falcon tube, and two volumes of ice-cold ethanol added. The contents were mixed by gentle inversion, and then the DNA was spooled out using either a fine glass rod or a fine plastic sterile loop. The spooled DNA was dipped into an eppendorf containing 70% ethanol (to wash the DNA and to remove any salts). The DNA was transferred to a labelled screw capped eppendorf, left to dry and then re-suspended in 1ml distilled water. To assess quantity and quality, an aliquot was diluted 1:50 and analysed by spectrophotometry at 260 and 280nm.

2.1.2 DNA Extraction from Cell Lines

DNA was extracted from cell lines using a high salt method, avoiding the use of phenol. To pellet the cells, 50ml Falcon (Greiner) tubes were spun at 1000rpm for 10 minutes. The supernatant was removed and the cells washed twice in phosphate buffered saline (PBS), before finally removing the supernatant. The cells (approximately $5x10^7$) were re-suspended in 15mls SE buffer, and 50µl of 10mg/ml RNase A (Advanced Biotechnologies) added to the tube before incubation at 37°C for 1 hour to degrade any RNA. Proteinase K was then added to a final concentration of 200µg/ml and the tubes left overnight at 55°C to degrade the protein. 4.5mls of pre-warmed 5M NaCl was added to the tubes to give a final concentration of 1.5M NaCl. 20ml chloroform (Merck) was then mixed in by rotation for 30 minutes before centrifugation at 2000rpm for 10 minutes. The aqueous layer was transferred to a new tube and an equal volume of isopropanol (BDH) mixed in for 5 minutes to allow the DNA to precipitate. After spooling out the DNA, it was washed in 70% ethanol for a minimum of one hour to remove any remaining salt from the DNA. The DNA was then re-suspended in distilled water and subjected to spectrophotometry to assess quality and quantity.

2.1.3 DNA Extraction from Paraffin Embedded Tissue

Paraffin blocks were cut to give 10µm sections on non-coated slides. The slides were de-waxed in xylene (BDH) for 10 minutes, followed by two washes of 10 minutes each in 100% ethanol (BDH). Using a haematoxylin and eosin stained slide as a guide for the area to be micro-dissected, the slides were scraped with a needle into an appropriate amount of digestion buffer (4.45ml dH_20, 500µl 10x magnesium-free PCR buffer (Perkin-Elmer), 50µl 20mg/ml Proteinase K (Merck)), depending on the size of the lesion. The tubes were vortexed and placed at 55°C for 1-3 days, with intermediate vortexing. The Proteinase K was de-activated by heating the tube to 95°C for 10 minutes and the tubes spun for 15 minutes at 13000rpm in a microfuge. The supernatant containing the DNA was transferred to a fresh tube and ready to be used.

2.1.4 DNA Extraction from Fresh Frozen Tissue

DNA was extracted from fresh-frozen tissue using the QIAamp tissue kit (Qiagen) which is specified to allow up to 40µg of DNA to be extracted from 25mg of soft tissue. The

manufacturer's protocol was followed. Briefly, the tissue was cut into small pieces, lysed, precipitated with ethanol and added to a spin column to which the DNA bound. After several washes, the DNA was eluted from the column with distilled water.

2.1.5 DNA Extraction from Agarose Gels Using Geneclene® SPIN Kit

DNA was purified from agarose gels using the above kit. This system relies on a silica matrix that binds to single and double stranded DNA, from which the DNA is eluted at the end of the process in water. Manufacturer's instructions were followed.

2.1.6 DNA Extraction from Clones

Isolation of DNA from P1 artificial chromosomes (PACs) was achieved using the Plasmid Midi Kit (Qiagen), following the protocol designed for the isolation of BAC (Bacterial Artificial Chromosomes) DNA. Stabs were streaked onto agar containing 25μg/ml Ampicillin and grown overnight at 37°C. Single colonies were inoculated into starter cultures of 5ml LB medium containing 0.5μl 25mg/ml Ampicillin and left to grow overnight with vigorous shaking (~250rpm). The manufacturer's instructions for the kit were then followed.

2.2 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is used to amplify regions of target DNA, and can be used provided as least part of the target nucleotide sequence is known. Portions of the sequence which flank the desired target are used to design two synthetic DNA oligonucleotides, one complementary to each strand of the DNA double helix. These oligonucleotides serve as primers for *in vitro* DNA synthesis, which is catalysed by a thermostable DNA polymerase, with the primers determining the ends of the amplified DNA fragment. The Primer3 program was used to design primers (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCRs were usually performed in either 25μl or50μl volumes, although volumes could be scaled up and down as necessary. A typical 25μl PCR reaction would be:

2.5μl 10x Mg^{2+}-free PCR buffer (Promega)

1.5μl Mg^{2+} (@ 25mM, giving final concentration of 1.5mM) (Promega)

1µl dNTPs (deoxyribonucloside triphosphates @ 2.5mM, giving final concentration of 0.1mM)(Pharmacia)

0.5µl Forward Primers (@ 20mM)

0.5µl Forward Primers (@ 20mM)

0.25 Taq DNA Polymerase (made in-house)

10-50ng DNA

Volume made up to 25µl with sterile dH_20

The DNA was aliquoted separately into microtitre plates (Advanced Biotechnologies), and then the PCR master mix was made up with the remaining components, vortexed briefly and added to the plate containing the DNA. The plate was then sealed with a heat plate to prevent evaporation during thermocycling. Three main stages comprised the PCR - first denaturation into single stranded DNA, followed by annealing, allowing the primers to find and anneal to the target sequence, then extension of the primers along the target sequence. A typical PCR reaction consisted of an initial denaturation of 94°C for 5 minutes, then 30-35 cycles each of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then a final extension step of 72°C for 10 minutes, usually performed on Tetrad PCR machines (MJResearch). The annealing temperature was optimised according to the T_m of the primers. When standard conditions failed to amplify the target DNA, a touchdown technique was employed, with the annealing temperature of 70°C decreasing by 0.5°C each cycle, for 19 cycles, then kept at 52°C but increasing the length of the cycle by 1 second per cycle for 19 cycles.

2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis separates DNA molecules according to their size and was most frequently used for checking for the presence of PCR products. Agarose was prepared by boiling a mixture of agarose powder (Gibco BRL) in 1XTBE, at concentrations of 1-3% depending on the size of the DNA fragments. When cooled to approximately 50°C, ethidium bromide (10mg/ml) (Pierce) was added and mixed in to a final concentration of 0.25µg/ml. The ethidium bromide intercalates with the DNA and fluoresced under ultraviolet light, allowing visualisation of the DNA fragments. Molten agarose was poured into a gel-casting tray with a comb in position and left to set. The comb was then removed; the gel was placed in a running tank and then covered with a running buffer of 1xTBE. 5µl of DNA was combined with 2µl of tracking dye and loaded into each well. 10µl of 1Kb ladder was loaded in the final well to allow sizing of the fragments and the gel electrophoresed at 100-130V for

10-30 minutes. Visualisation of the DNA was then performed by placing the gel on a UV transilluminator (260nm) and photographs taken using a UV products camera.

2.4 Purification of PCR Products

Prior to sequencing, PCR products were purified using the Qiaquick PCR purification spin columns or the Qiaquick 96-well format columns (Qiagen) according to the manufacturer's instructions. The purification separated the target DNA from excess dNTPs and primers that may have interfered with subsequent downstream experiments. For example, if too much unbound primer were present in purified PCR products, the chances of primer-dimer formation increased. The sequencing reactions performed on such templates would mainly consist of primer sequences and not the PCR product.

2.5 Sequencing Protocols

2.5.1 Direct Sequencing of PCR Products

Sequencing of PCR products was performed using the Big Dye Terminator sequencing mix (Applied Biosystems) which incorporates base-specific fluorescent nucleotides, utilising the dideoxy chain-termination method (Sanger, 1981). This method is based upon the enzymatic incorporation of dideoxyribonucleoside triphosphates in which the deoxyribose 3'-OH normally present is missing. When these modified nucleotides are incorporated the addition of subsequent nucleotides is blocked, which leads to fluorescent DNA 'ladders' of differing lengths which can then be separated on polyacrylamide gels. Sequencing reactions were made as follows:

8µl Big Dye Terminator mix (PE Applied Biosystems)

0.5µl primer (either forward or reverse, as used in the PCR)

6.5µl dH_20

5µl purified PCR product

Cycle-sequencing was performed in a PCR machine with an initial denaturation at 94°C for 4 minutes, followed by 25 cycles of 94°C for 30 seconds, 50°C for 10 seconds and 60°C for 4 minutes, with a final extension of 60°C for 7 minutes. The sequencing products were cleaned up to remove excess salts and big dyes using the Qiaquick columns as described in 2.4 and spun down in a vacuum centrifuge until dry. The products were then re-suspended in 3µl of microSTOP loading buffer (Perkin-Elmer), denatured at 94°C for 4 minutes, and run on an

ABI377 Sequencer (Applied Biosystems) on 5% Severn Super Sequencing mix (Severn) polyacrylamide gels.

2.5.2 Sequence Analysis

Sequences were analysed using Semi-adaptive base calling and Sequencing Analysis Version 2.1 (Applied Biosystems). Database searching using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was performed to ensure the correct origin of the sequenced fragments. Alignment of sequences was performed using the Clustal V method based on a distance matrix (Higgins and Sharp, 1989) included in the MegAlign software module (DNASTAR). In addition, all sequences were examined by eye to look for heterozygous peaks that might not be detected by alignment tools.

2.6 Mutation Detection Techniques

2.6.1 Single Stranded Conformational Polymorphism Analysis

The rate of migration of single stranded DNA under non-denaturing conditions through a polyacrylamide gel is sensitive to secondary structure, and this structure in turn depends on the nucleotide sequence. If there are sequence differences between different strands of DNA, the secondary structure may be altered and this will be detected as a band of altered mobility on Single stranded conformational polymorphism (SSCP) analysis. SSCP was used to search for mutations such as point mutations (missense or nonsense), insertions and deletions in PCR products of up to 350 base pairs in length. Two main SSCP methods were employed – polyacrylamide gel electrophoresis (PAGE) combined with silver staining or capillary separation using an ABI3100 (Applied Biosystems).

2.6.1.1 SSCP Using Page and Silver Staining

PAGE was performed using either the Phast minigel system (Pharmacia) or using large self-poured plates (Bio-rad Protean II SSCP system). For the Phast system, 2µl of the PCR product was combined with 2µl of SSCP loading buffer (98% formamide (Amersham) containing 0.05% bromophenol blue (Sigma) and 0.05% xylene cyanol (Sigma), and denatured at 95°C for 5 minutes. Larger fragments were run on pre-cast 12.5% gels for 130-150 volt-hours, and smaller fragments separated on pre-cast 20% gels for 140-150 volt hours, with the running temperature generally 10°C or 15°C. Silver-staining was used to detect the

bands according to the manufacturer's instructions. For the self-cast large gels, the following mix was prepared and poured between two clean plates assembled with spacers:

19.8mls dH_20

8.2ml acrylamide:bis 35% (39:1)

8ml 5XTBE

2ml Glycerol (BDH)

The gel mix was polymerised with 300µl 10% w/v ammonium persulphate (APS)(Sigma) and 50µl TEMED (NNN'N'-tetramethylethylenediamine) (BDH). 5µl of PCR product was combined with 5µl SSCP loading buffer before being denatured and loaded on the set gels. To size the separated products, 5µl of 100 base pair ladder (Gibco) was combined with 5µl of SSCP loading buffer and loaded into the final well. The gels were then run at 150Mamps at room temperature for ~4 hours or overnight in the cold room (4°C). The gels were separated from the plates and then silver stained (shown in Solutions section). Any samples showing aberrant migration were re-amplified from the source DNA, purified using Qiaquick columns (Qiagen) and directly sequenced using the ABI Big Dye Terminator kit (Applied Biosystems).

2.6.1.2 SSCP Using the ABI 3100

The second technique for SSCP analysis utilised a capillary based method. PCR products were amplified with the forward and reverse primers fluorescently dye-labelled (FAM, TET or HEX). 5µl of diluted (1/50) PCR products were combined with 0.5µl internal size standard (Tamra 350, Applied Biosystems) and 11.5µl 310 loading buffer. The samples were denatured at 95°C for five minutes, plunged onto ice and then run on an ABI 3100 sequencer (Applied Biosystems), using 2% Genescan polymer containing glycerol (Applied Biosystems). SSCP was performed under two different temperature conditions (18°C and 24°C), according to manufacturer's instructions. Sequence changes within the fragments presented as a different pattern or altered size when compared to the size standard. Fragments showing both aberrant and normal migration were re-amplified using non-fluorescently labelled primers, purified using Qiaquick columns (Qiagen) and then sequenced in both forward and reverse orientations using the ABI Big Dye Terminator kit (Applied Biosystems). This 96-well format high throughput system allowed three fragments to be assessed simultaneously in the same well.

2.6.2 Denaturing High Performance Liquid Chromatography Using the WAVE® System

Denaturing High Performance Liquid Chromatography (DHPLC) is a chromatographic mutation analysis method based on temperature-dependant separation of DNA containing mismatched base pairs from a pool of PCR amplified DNA fragments. Mutation detection by DHPLC relies on the formation and separation of double-stranded DNA fragments that contain mismatched bases known as heteroduplex DNA. Heteroduplex DNA is generated by denaturing and re-annealing a mixed population of reference or 'wild-type' sample and 'mutant' DNA. The heteroduplex DNA fragments form as a result of base-pairing in the single-stranded, mutated DNA with a single-stranded 'wild-type' DNA. The two strands will not form hydrogen bonds at the mutation site because the base-pairs are mismatched, thus giving the heteroduplex different melting properties than the homoduplex. Using the WAVE® system, heteroduplexes can be resolved from homoduplexes. At a critical temperature (partially denaturing conditions), the mismatched bases in the heteroduplexes begin to separate, while the matched bases of the homoduplexes remain intact. Heteroduplex fragments elute earlier in the gradient, specifically the heteroduplex DNA is detected by the presence of additional 'peaks' or 'shoulders' in the resulting chromatogram. When compared to a reference or 'wild-type' DNA amplicon, these additional peaks signal the presence of a mutation.

DHPLC was done using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK). The same primer set was used as for F-SSCP except that no fluorescent labelling was used for this technique. To enhance the formation of heteroduplexes prior to analysis, the PCR products were denatured at 94°C and re-annealed by cooling to 50°C at a rate of 1°C per minute. DHPLC was carried out at the melting temperature predicted by Wavemaker (version 4.0) software (Transgenomic) with a 12% acetonitrile gradient over 2.5 minutes. Samples displaying aberrant dHPLC elution profiles were sequenced directly.

2.6.3 Protein Truncation Test

2.6.3.1 *In-Vitro* Translation of PCR Products

The protein truncation test (PTT) detects nonsense mutations – point mutations which result in a stop codon, or frameshifts which results in a downstream aberrant stop signal. PCR primers were designed with MYC, T7 RNA-polymerase binding site and RBS (ribosomal binding site) tags and an in-frame start codon on the forward primer. These latter tags enabled transcription and translation respectively. The tag added to the forward primers was as follows:

5'GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGAACAAAAATTAAT ATCGGAAGAGGATTTGAAT

PCRs were performed on cDNA using standard conditions to amplify the entire coding region with overlapping fragments, ensuring each fragment started in-frame. The products were then kept at -20°C until ready for use. To allow *in vitro*-coupled transcription and translation (IVTT), 15µl of the tagged PCR products were combined with the following reagents which had been carefully mixed together:

8µl Rabbit Reticulocyte Lysate
0.66µl TnT buffer
0.34µl amino acids minus methionine
0.34µl T7 polymerase
0.66µl ^{35}Smethionine
0.33µl RNase inhibitor
4.67µl dH_20

The mix was then incubated for 1or 2 hours at 30°C on a PCR machine, with the plate covered by 3MM paper to stop any isotope vapour escaping.

2.6.3.2 Electrophoresis of Translated Products

For the electrophoresis of the resulting 'proteins', two polyacrylamide gel mixes were required – one to stack the proteins and one to resolve the proteins. Aberrant stops were detected as a truncated 'protein' band when subjected to PAGE. To prepare enough 12% resolving gel mix for two gels, 8ml acrylamide (30%, 37.5:1) (Severn Biotech) was mixed with 7ml distilled water and 5ml lower buffer mix. Two clean plates were assembled with a

gasket acting as a spacer, and bulldog clips holding the assembly together. 100µl of 20% w/v ammonium persulphate (APS) and 20µl of TEMED were added to the lower gel mix, swirled gently and then poured into the glass plate assembly. 500µl of water was added on top of the resolving gel to ensure a straight edge was obtained. Whilst the resolving gel polymerised, the 1.125% upper (stacking) gel mix was made by mixing 2.5ml acrylamide (30% 37.5:1) with 8.5ml distilled water and 2.5ml upper buffer. The water was poured away from the lower gel, and 500µl of the un-polymerised upper gel mix added in its place to allow good contact between the two gels. A 12-well sharks-tooth comb was added at an angle to allow the pouring of the upper gel. The upper gel mix was poured away from the lower gel, and 20µl TEMED and 100µl 20% APS added to the remaining upper mix before pouring it onto the lower gel. The comb was straightened and excess gel wiped away. 1.5-2 inches of PTT running buffer was added to the running tank. Once the gels had set (10-20 minutes), the clips and gasket and comb were carefully removed. The wells were rinsed and straightened using a syringe filled with running buffer. The two plates were clamped into the running tank and the reservoir filled with running buffer. 10µl of sample buffer (9µl bromophenol blue plus 1µl 1M DTT) was added to each well, the plate was sealed and covered with 3MM to stop any vapour escaping and then denatured at 95°C for 5 minutes. 15 µl of each sample was carefully loaded into wells 1-11, avoiding air bubbles, and then 7.5µl of multicoloured protein marker (NEN) was added to the final well for orientation. The gel was run at 60m amps for 1-1.5 hours or until the loading buffer was visible near the bottom of the gel.

2.6.3.3 Fixing, Drying and Exposure of the Gel

Following electrophoresis, the running buffer was carefully disposed of in the hood and the plate assemblies were placed on paper towels to ensure that no isotope was left on the surface. The plates were separated using forceps and the gels placed into fixing solution until the bromophenol blue in the sample buffer had turned green. In the meantime, the gel dryer was warmed to 80°C with a piece of 3MM paper in. The gels were then transferred onto a piece of 3MM on the bench added to the 3MM in the dryer. The gels were dried under vacuum forl hour and then taped into an X-ray cassette. In the dark room, Kodak film was laid over the gels and left overnight before developing in an automatic developer.

2.7 Sterile Lymphocyte Separations

Separations were performed so that the lymphocytes could be established as permanent cell lines by Epstein - Barr virus transformation. 25ml blood was collected in Falcon tubes containing 25ml sodium citrate medium. The contents of the Falcon were poured into a 250ml flask, and the tube rinsed with 4ml filtered RPMI which had been brought to room temperature. Approximately 25 sterile glass beads (BDH) and 0.6ml 1M $CaCl_2$ (BDH) were added to the flask and defibrination started immediately for 15 minutes at 250rpm on a gyratory shaker. 20ml RPMI was added to the flask and the defibrinated blood divided into two tubes, layering carefully over 15ml lymphoprep (Robbins Scientific). The tubes were spun at 1800 rpm for 20 minutes in a centrifuge with a swing out centrifuge rotor, with the speed carefully brought up and down. The interface was then transferred to a new Falcon and spun at 2300rpm for 10 minutes. The pellet was washed in 20ml RPMI then re-spun at 2300rpm for 10 minutes. The pellets were re-suspended in 2ml freeze mix, divided into two labelled cryotubes (Corning) and placed at -80°C overnight. The lymphocytes were then stored in liquid nitrogen until ready for transformation.

2.8 Tissue Culture

2.8.1 Feeding Cell Lines

Once the lymphocytes had been transformed and returned as a growing culture, the cells would either need feeding or spinning down for DNA/RNA/protein extraction as described above. The cells were examined under a microscope to determine the viability and density. If the cells were to be left to continue to grow but were confluent, an equal volume of growth medium was added to the flask and left at 37°C overnight in 10% $C0_2$. The cells were then re-examined and either left to grow or spun down for required protocol.

2.8.2 Freezing Down Cells To Replace Stocks

To ensure the cell lines remain a permanent resource, an aliquot of the growing cultures was always frozen down. 50ml of the growing culture was spun at 2000rpm for 5 minutes, the supernatant removed and the tube inverted to dry. The pellet was then fully re-suspended in 2ml freeze mix, and aliquoted into sterile cryotubes labelled with the cell line name, date,

volume spun down and initials. The tubes were frozen at -70°C overnight then stored in liquid nitrogen.

2.9 Comparative Genomic Hybridisation

Comparative genomic hybridisation (CGH) is a useful tool for performing a genome wide scan of chromosomal loss and gains in a tumour (Kallioniemi *et al.*, 1992). A mixture of DNA from malignant and normal cells are differentially labelled with red or green fluorochromes and then hybridised onto metaphase spreads. Images of 5-10 metaphases are captured and quantification of the fluorescence ratios performed using a digital image analysis system. The relative ratios of red and green are then compared. Regions of genetic material which have been lost during tumour progression will show as red, and regions which have been gained will show as green. The thresholds of detection for CGH are regions greater than 10Mb for loss of genetic material, and gains of 2Mb or more if the region is amplified five times.

2.9.1 Nick Translation and Precipitation Of Probe

One μg of tumour DNA or test DNA was labelled with FITC–12-dUTP (Vysis) or Texas Red–5-dUTP (Vysis) respectively. This was achieved by mixing the DNA, 1μl of the relevant fluorochrome, 5μl dNTPs, 10μl DNA polymerase I/DNase mix, and 1μl DNA polymerase I and distilled water to make a total volume of 50μl. The mix was incubated at 15°C for 2 hours, and then left on ice while 5μl was run on a 1% agarose gel. Probe fragments forming a smear ranging in size between 500 and 2000 base pairs were the best length for smooth hybridisations. The reactions were stopped by adding 5μl of 0.5M EDTA and could be stored at -20°C until ready for use. The tumour and test DNAs were then combined in an Eppendorf tube and mixed with 50μl of human Cot-1 DNA, 0.1 volume 3M sodium acetate, and 2 volumes cold absolute ethanol. The DNA was precipitated on dry ice for 30 minutes or overnight at -20°C. After spinning at 15000rpm, the supernatant was carefully removed and the pellet left to air dry. The pellet was then re-suspended in 10μl of hybridisation mix and denatured at 75°C for 5 minutes. The DNA was left to pre-anneal at 37°C for 30 minutes to 1 hour whilst the slides were prepared for hybridisation.

2.9.2 DOP-PCR Labeling Of Tumour DNA for CGH

Degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) labelling of tumour DNA was performed when there was not sufficient or good quality DNA to nick translate, usually when the DNA was extracted from paraffin embedded tissue. The PCR-labeling used a degenerate primer (sequence 5' ccgactcgagnnnnnnatgtgg 3') and had two stages, initial low stringency cycles, where the specific bases at the 3' end of the oligonucleotide theoretically primed every 4 kb along the template DNA, and then an increased number of cycles with high stringency, whereby the oligonucleotide 'tailed' DNA from the initial cycles was amplified. Further DOP-PCR, with differential fluorescent nucleotides incorporated into the PCR reaction was then performed to label the tumour and normal DNA with their respective fluorochromes. Labeled DNA was then precipitated and hybridised to metaphase spreads, using the same protocols as those for nick-translated DNA. The first round DOP-PCR reactions were set up as follows;

2.5µl 10 X Mg^{2+} free PCR buffer

2.5µl dNTPs (@ 2 mM, giving final concentration of 200µm)

0.5µl DOP 6MW primer (at 100µm giving concentration of 2.0µ M)

4µl Mg^{2+} (@25mM giving concentration of 4mM)

0.5µl Taq polymerase

15µl tumour or normal DNA

Volume made up to 25µl with dH_20

The DOP-PCR conditions were as follows: 1 cycle of 9 mins @ 94°C, 8 cycles of (1 min @ 94°C, 1.5 mins @ 30°C, 3 mins @ 72°C) then 25 cycles of (1 min @ 94°C. 1 min @ 62°C, 1.5 min @ 72°C) followed by a final extension of 8 mins at 72°C.

The second round labeling DOP-PCR experiments were set up as follows;

5µl 10 X Mg^{2+} free PCR buffer

5µl labeling dNTPs (2 mM each of dATP, dCTP and dGTP, 0.5mM dTTP, giving final concentration of 200µm)

0.5µl DOP 6MW primer (at 100µm giving concentration of 2.0µ M)

8µl Mg^{2+} (@25mM giving concentration of 4mM)

1µl Taq polymerase

10µl of first round DOP-PCR tumour or normal DNA

Volume made up to 50µl with dH_20

The labeling DOP-PCR conditions were as follows: 1 cycle of 4 mins @ 94°C, then 25 cycles of (1 min @ 94°C. 1 min @ 62°C, 1.5 min @ 72°C) followed by a final extension of 8 mins at 72°C.

2.9.3 Denaturing the Slide and Hybridisation

Each batch of slides, whether commercial (Vysis) or made in-house, had a pre-determined optimal denaturing time. Prior to denaturing, slides were examined under the microscope to ensure they were suitable for hybridisation, with many, distinct metaphases free of cytoplasm being ideal. The slides were then denatured on a hotplate at 73°C with denaturation solution under a 22 mm X 50 mm cover slip. The cover slip was then flicked off and the slides placed in ice cold 70% ethanol for three minutes, and then dehydrated through an ethanol series for three minutes each. The slides were air dried and then ready for use. For the hybridisation, the slides were placed on a hotplate at 37°C and 10µl of a denatured probe added to each half of the slide. Each probe was covered with a 22 X 22mm cover slip, sealed with rubber cement and sealed in a moist chamber for 48-72 hours at 37°C.

2.9.4 Post-Hybridisation Washing Of the Slides

Following hybridisation, the cover slip was removed from the slides and the slides subjected to 3 X 5 minute washes in 50% formamide/2XSSC at 42°C, then 3 X 5 minutes in 2XSSC, again at 42°C. The slides were then subjected to a 5 minute wash at room temperature in SSCT, whilst shaking gently, before dehydrating through an ethanol series (70%, 95%, and 100%) and being left to air dry. The slides were then mounted in DAPI (approximately 20µl under a 22 X 50mm coverslip), and either stored in a cardboard folder at 4 °C or captured immediately.

2.9.5 Image Acquisition and Analysis

5-10 metaphases per experiment were captured using an epifluorescence microscope (Applied Imaging) equipped with a triple-color epifluorescence filter set (selective for the fluorochromes DAPI, FITC, and rhodamine) in combination with a cooled CCD camera (Quantix Photometrix). Images were captured using Quips software (Vysis). The metaphases

were karyotyped using the digitally inverted DAPI image which gave a G-banded pattern. After karyotyping the relative intensities of the red and green signals were analysed and an average obtained for multiple metaphases. CGH experiments were considered successful if enough fluorochrome had been incorporated to give smooth intense color that was not granular in appearance.

2.10 Immunohistochemistry

2.10.1 Slide Preparation

Five micrometre sections were placed on slides. Slides were dewaxed in a xylene to water series. Racked slides were immersed in xylene for 5 minutes then 100% ethanol for three minutes, 90% ethanol for three minutes, and 70% ethanol for three minutes. Endogenous peroxide activity was then blocked with 2% H_2O_2 by immersion for 20-30 minutes.

2.10.2 Exposing Antigen and Applying Primary Antibody

Citrate buffer was heated to boiling in a pressure cooker and slides were placed in the pressure cooker on full pressure for four minutes. Slides were allowed to cool in the citrate buffer then rinsed in PBS pH 7.45. PBS was then tipped off and the nonspecific immunoglobulins blocked with normal serum at a dilution of one in 5 for 30 minutes.
For polyclonal antibodies (such as MSH2) 15µl goat serum is diluted in 985µl PBS.
For monoclonal antibodies (such as MLH1) 15µl horse serum is diluted in 985µl PBS.
Primary antibody (75µl of diluted mixture) is then added at a concentration of 1/100 (MSH2, MLH1, MSH6, beta-catenin and SMAD4) or 1/1000 (P53) and the slides incubated at 4°C for 24 hours.

2.10.3 Applying Secondary Antibody and Counterstaining

Sections were rinsed in PBS for five minutes. They were then incubated in biotinylated secondary antibody at a dilution of 1/200 for 30 minutes.
For polyclonal antibodies: 15µl goat serum + 5µl Anti-rabbit + 980µl PBS per 1mL.
For monoclonal antibodies: 15µl horse serum + 5µl Anti-mouse + 980µl PBS per 1mL.

Next the slides were incubated in Streptavidin-Peroxidase C188 (Dako E354) 1/300 for 30 minutes then washed twice in PBS for five minutes. Peroxidase substrate (DAB) was then applied for two minutes watching progress carefully to avoid overstaining. Slides were then washed in water and counterstained in Haematoxylin for two minutes. Finally slides were dehydrated through a series of ethanol 70%, 90%, 100% and xylene and then mounted in DPX mountant.

2.11 Flow Cytometry

Flow cytometry is used to measure the DNA content of cells amongst many other applications. This can allow estimation of the degree of polyploidy or aneuploidy of a population of cells. It can also give information about the cell cycle. The DNA-binding fluorochrome propidium iodide was used for all analysis in this project.
All cells in the G1 phase will take up the same amount of dye and will fluoresce in a single channel however there will be minor conformational variations in the DNA leading to different amounts of dye being taken up. This is quantitated by using the coefficient of variation of the G1 peak. The lower the co-efficient of variation, the better changes such as aneuploidy can be assessed. The DNA content is being inferred by the amount of fluorochrome binding and the scatter pattern of light as cells pass through the reading laser. Larger cells cause greater scatter of light and this can be quantitated.

2.11.1 Isolating Nuclei from Paraffin Embedded Tissue

40μM sections were cut from paraffin embedded archival tissue. These were placed between layers of 3MM Whatman paper and placed in a small histopathology cassette. These were dewaxed in xylene for one to 12 hours if many samples were being processed. The samples were then re-hydrated through an alcohol series 95%, 70% and 50%. The 95% ethanol was changed once to remove all traces of xylene. The tissue was left in each solution for 20 to 30 minutes. Cassettes were then rinsed in ddH_2O for 15-20 minutes twice. The re-hydrated tissue section was removed from the cassette and transferred to a 1.5ml eppendorf tube. One ml of 0.4% pepsin solution at 37°C was added and the tissue allowed to digest for 30 minutes. Digestion was stopped by adding 0.2% glycine in 2xPBS. The nuclei were then spun down and washed with PBS twice.

2.11.2 FACS Analysis

Cells were resuspended in 100ul RNase (Sigma; 100ug/ml) and 400ul propidium iodide (Sigma; 50ug/ml) and incubated at room temperature for 30 minutes. Samples were analysed using a FACS Calibur (Becton Dickinson, San Jose, Ca) with gates being set on forward and side scatter to exclude debris and PI signal area and width to exclude cell doublets. Propidium iodide fluorescence was collected above 670nm and samples were analysed using CellQuest software (BD).

2.12 Cloning of PCR Products

2.12.1 Ligation of PCR Products into Cloning Vectors

PCR was performed using 100 ng of template DNA. This was run on 2% LMP agarose gel at 60V for 90 minutes. The resulting band was then cut out over ultraviolet light and the DNA purified. PCR products were cloned directly into the pGEM® Easy vector using the pGEM®-T Easy Vector System (Promega) following the manufacturers instructions.

2.12.2 E.coli Transformation

A 14ml FALCON® tube (Becton Dickinson) was chilled on ice for each transformation. 50-100µl of competent cells (Stratagene) was added. Two microlitres of β-mercaptoethanol were added and the mixture left on ice for ten minutes. Two microlitres of each ligation mix was added to a tube of cells which was then incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds then returned to the ice bath. 0.9ml of preheated 42°C NZY^+ broth was added and the tubes incubated at 37°C for one hour with shaking at 225-250 rpm. 100µl of each sample was plated on an agar plate containing 0.2mM IPTG (Isopropylthio-β-D-galactoside) and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) 0.3mg/ml and incubated overnight at 37°C.

2.12.3 Generation of Single Stranded DNA and Purification of Plasmid DNA

Each clone/colony to be analyses was picked of the plate and inoculated into 5ml of Luria broth containing Ampicillin (Sigma) at 50μg/ml. The cultures were incubated overnight at 37°C with shaking at 300rpm. The following morning the cells were pelleted by spinning at 13000rpm for 1 minute. Preparation of the DNA is performed as follows using a QIAprep® Miniprep kit (Quiagen). Bacterial cells containing plasmid then were lysed under alkaline conditions and the lysate neutralized and purified by passing through a filter before adsorption on a silica-gel membrane in the presence of a high salt concentration. Endonucleases and salts were removed by washing in kit buffers and DNA eluted in water. 1.5μl of each DNA was then used in a sequencing reaction for the region of interest and run on the ABI 377 or ABI 3100 using standard protocols.

2.13 Solutions and Media For Molecular Techniques

1M $CaCl_2$

14.7g of Calcium Chloride (BDH) made up to 100ml with dH_20

Cell lysis buffer

1ml buffer (2x bromophenol blue, with SDS+sucrose)

800μl dH20

200μl 1M DTT (makes 0.1M DTT)

Citrate Buffer

10L ddH_20

20g Citrate acid

8g NaOH pellets

Adjust to pH 6.0

DAB

5mg DAB (Sigma D5637)

10ml PBS

20μl H_2O_2

Denaturing solution:(for 2 gels)

262.93g sodium chloride

60g sodium hydroxide pellets (BDH) - made up to 3L with dH_20.

3100 dilution and running buffer

12.5ml 20xTBE

25ml glycerol (BDH)

Made up to 250ml with dH_20 and stored at 4°C.

100XDenhardt's solution

10g ficoll 400 (Pharmacia)

10g polyvinlypyrrolidine (BDH)

10g bovine serum albumin (Sigma)

Made up to 500ml with dH_20, stored at -20°C.

0.5M Ethylenediamine tetraacetate (EDTA)

93g EDTA (BDH)

10g sodium hydroxide pellets (BDH)

400ml dH_20

pH adjusted to 8.0, made up to 500ml with dH_20 and autoclaved.

Ethidium bromide (10mg/ml)

0.1g of ethidium bromide (Pierce) dissolved in 10ml dH_20. Stored in dark.

Fixing solution :10% ethanol/5% acetic acid

100mls 100% ethanol (BDH)

50mls acetic acid (BDH)

850mls dH_20

Freeze mix (90%FCS/10%DMSO)

9ml foetal calf serum (GibcoBRL))

1ml Dimethyl sulphoxide (BDH)

5% stock Genescan polymer

7.14ml GS polymer (Applied Biosystems)

2.86ml 3100 dilution buffer

2% Genescan polymer

2ml 5% stock GS polymer

3ml 3100 dilution buffer

Growth Medium 10% FCS/RPMI

180mls RPMI (one bottle)

20mls FCS (one vial)

2% H_2O_2

600mL Methanol

36mL 30% H_2O_2

High stringency wash (0.2XSSC/0.1%SDS)

10mls 20XSSC

10mls 20% SDS - up to 1L with dH_2O

Hybridisation solution (500ml)

211.25mls dH_2O

150mls 20XSSC

100mls 50% Dextran Sulphate

12.5mls 10% SDS

25mls 100X Denhardts solution

1.25mls 10mg/ml Salmon Sperm DNA (Amersham)

310 Loading buffer

1100µl deionized formamide (Amersham)

50µl 0.3N (0.6g/50ml) sodium hydroxide

Low stringency wash (2XSSC/0.1% SDS)

100mls 20XSSC

10mls 20% SDS - up to 1L with dH_2O

Lower buffer mix (pH 8.8)

90.75g Tris (1.5M)

20ml 10%SDS (0.4%)

made up to 500ml with dH_20 and pH adjusted to 8.8 with HCL.

Luria Broth (LB)

10g sodium chloride

5g bacto yeast extract

10g bacto-trytone

900ml dH_20

pH adjusted to 7.0 with sodium hydroxide, made up to 1L with with dH_20, and autoclaved.

LB agar plates

LB medium prepared as above, and 15g/Litre bacto-agar added before autoclaving.

Nuclei lysis buffer(50ml)

10mM Tris (500µl 1M)

400mM NaCl (4mls 5M)

2mM EDTA (200µl 0.5M) (BDH)

45.3mls dH_20

Neutralising solution:(for 2 gels)

262.93g sodium chloride (Sigma)

181.5g Trizma base (Sigma) - made up to 3L with dH_20.

PBS

10dL ddH_20

80g NaCl

2g KCl

11.5g Na_2HPO_4

2g KH_2PO_4

Adjust to pH 7.5

Pepsin solution:(For FACS analysis)

0.4% pepsin in 0.1M HCl pH1.8 -(pre-warm 200ml 0.1M HCl, add 0.8g pepsin JUST before use).

Prehybridisation solution: (500ml)

311.25mls dH_20

150mls 20XSSC

12.5mls 10% SDS

25mls 100X Denhardts solution

1.25mls 10mg/ml Salmon Sperm DNA (Amersham)

Proteinase K buffer (stored at 4°C)

2mM Na-EDTA (200µl 0.5M solution)

1% SDS (5ml 10%SDS)

44.8mls dH_20

Proteinase K solution

2mg Proteinase K (Merck) in 1ml EDTA/SDS buffer.

PTT running buffer

3g Tris (Sigma)

14.4g glycine (BDH)

10ml 10% SDS, made up to 1L with distilled water

3% w/v skimmed milk powder

12g skimmed milk powder (Premier Brands) in 400mls PBS

5% w/v skimmed milk powder

20g skimmed milk powder (Premier Brands) in 400mls PBS

SE buffer

75mM NaCl (7.5mls 5M)

25mM EDTA pH 8.0 (2.5mls 0.5M)

1% SDS (50mls 10%)

made up to 500ml with dH_20 and sterilised through 0.2 μ pore filter.

Silver Staining solutions/protocol

2 x 3 minutes in **fixing** solution: 10% Ethanol(100ml/L), 0.5% acetic acid (5ml/L)

1 x 15 minutes in **staining** solution: 0.1% (1g/L) silver nitrate ($AgNO_4$) (Sigma)

2 x 1 minute in dH_20

1 x 20 minute in **developing** solution: 1.5% (9.374g/L)sodium hydroxide (BDH),

0.1% (1ml/L)Formaldehyde (BDH), prepared immediately before use.

1 x 10 minutes in **stop** solution:0.75% (7.5g/L) sodium carbonate (NA_3CO_4) (BDH).

Gentle agitation required with all solutions.

3M Sodium acetate

61.52g sodium acetate (BDH)

200ml dH_20

pH adjusted to 6.0, made up to 250ml with dH_20, and autoclaved.

5M sodium chloride (NaCl)

73.1g sodium chloride (BDH)

made up to 250ml with dH_20 and autoclaved.

Sodium dodecyl sulphate (SDS)

10% w/v volume SDS (BDH) in sterile dH_20.

5xTBE

54g Tris base (Sigma)

27.5g Boric acid (BDH)

20ml 0.5M EDTA pH 8.0

Made up to 1 litre with dH_20 and autoclaved.

Transfer buffer(20XSSC)

88.23g Tri-sodium citrate (BDH)

175.32g sodium chloride (Sigma)- made up to 1L with dH_20 (final pH7-8).

1M Tris

60.55g Tris base (Sigma)

400ml distilled water, pH adjusted to 8.0, then made up to 500ml dH_20 and autoclaved.

Upper buffer mix (pH 6.8)

30.25g Tris (0.5M)

20ml 10%SDS (0.4%)

Made up to 500ml with dH_20, pH adjusted to 6.8 with HCL.

2.11 solutions for cytogenetic techniques

Denaturing solution for slides

70% formamide (700µl)

2XSSC (100µl 20XSSC)

200µl dH_20

Detection reagents

For Biotin labelled probes – avidin-FITC (Vector labs) diluted 1/500 with SSCT

For digoxygenin labelled probes – anti-Dig-Rhodamine (Boehringer Mannheim) diluted 1/100 in SSCT.

Ethanol series for dehydration

70% - 700ml absolute ethanol and 300ml dH_20

95% - 950ml absolute ethanol plus 50ml dH_20

100% - absolute ethanol

2XSSC (10% v/v 20XSSC)

50ml 20XSSC

450ml dH_20

SSCT

4X SSC (100ml 20XSSC)

0.05% Tween-20 (250µl)

pH 7.0, made up to 500ml with dH_20

SSCTM

5% skimmed milk powder (25g)

made up to 500ml with SSCT

2.14 Abbreviations

aa	– amino acid
AAPC	– Attenuated adenomatous polyposis coli.
A	- adenine
APC	- adenomatous polyposis coli
BER	- base excision repair
BMPR1A	- bone morphogenic protein receptor 1A
bp	-base pairs
CD	- Cowden disease
CGH	- comparative genomic hybridisation
CRAC1	-colorectal cancer haplotype 1
CRC	– colorectal cancer
cDNA	– copy DNA
C	- cytosine
dH_20	– distilled water
DNA	- deoxyribonucleic acid
FAP	- familial adenomatous polyposis
FDR	– first degree relative
GI	- gastrointestinal
G	- guanine
HMPS	– hereditary mixed polyposis syndrome
hMLH1 or MLH1	– Human Mut L homologue 1.
hMSH2 or MSH2	– Human Mut S homologue 2.
hMSH6 or MSH6	– Human Mut S homologue 6.
hMLH3 or MLH3	– Human Mut L homologue 3
hMTH or MTH1	– Human Mut T homologue.
hMYH or MYH	– human Mut Y homologue
HNPCC	– Hereditary non-polypsis colorectal cancer
HPP	– hyperplastic polyp.
JPS	–juvenile polyposis syndrome
k-ras	– Kirsten Ras
Kb	– kilo base pairs
LOH	– loss of heterozygosity

MA	– multiple adenoma (phenotype)
MAP	– MYH associated polyposis.
Mb	– mega base pairs
MMR	– mismatch repair.
MSI	– microsatellite instability
MSI-H	– High level microsatellite instability
MSI-L	– Low level microsatellite instability
MSS	– Microsatellite stable
nt	- nucleotides
OGG1	– Human Mut M homologue
8-oxoG	- 8-oxo-7,8-dihydro2'deoxyguanosine
PCR	– polymerase chain reaction
PJS	- Peutz-Jeghers Syndrome
PTT	– protein truncation test
RNA	- ribonucleic acid
SA	- serrated adenoma
SSCP	– single stranded conformational polymorphism
TA	– tubular adenoma
T	– thiamine.
TVA	– tubulovillous adenoma
U	- uracil

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All genes have been italicised throughout, whereas protein and phenotypic symbols are shown in plain text.

Chapter 3 Candidate Gene Analysis in Multiple Adenoma Patients

3.1 Introduction

Patients with a multiple colorectal adenoma phenotype develop from 3 to 100+ colorectal adenomas throughout their lifetime. These may be synchronous or metachronous. The condition can appear to be sporadic/recessive or dominant within families. There appears to be no significant increase in extra-colonic manifestations or other cancers aside from those families found to have *APC* mutations. About 5% of multiple adenoma cases are due to mutations in the extreme 3' and 5' ends of the *APC* gene with some AAPC mutations also having been found in exon 9 (Spirio, Olschwang et al. 1993; Lamlum, Al Tassan et al. 2000). The phenotype in these families tends to vary widely from person to person. It is possible for persons with *APC* mutations generally associated with AAPC to develop a florid polyposis phenotype. A small proportion of such cases may also be due to HNPCC although the incidence of adenoma formation does not seem to be much higher than normal in MMR gene mutation carriers. The majority of patients who present in this manner have no detectable germline mutations. A candidate gene screen has therefore been undertaken in a group of multiple adenoma patients in an attempt to explain the germline predisposition in more of these patients. Candidate genes were selected o the basis of their integral role in pathways already known to be involved with colorectal carcinogenesis in both hereditary and sporadic forms.

The *APC* gene, known to be involved in both sporadic and familial adenoma formation is an integral part of the Wnt signaling pathway (Vogelstein, Fearon et al. 1988; Lamlum, Papadopoulou et al. 2000; Rowan, Lamlum et al. 2000). As described in chapter 1, the APC protein interacts with GSK3β, axin and conduction to form a complex which mediates proteosomal degradation of cytosolic beta-catenin, preventing its translocation to the nucleus and activation of a variety of transcription response elements. *APC2*, an *APC* homologue located on chromosome 19p 13.3 has also been shown to interact with beta-catenin binding domains, armadillo repeats and regulatory domains but lacks the COOH terminus domain which mediates microtubule binding. Human *APC2* is a 2301 aa protein which is expressed in many different tissues including colon, central nervous system, breast and ovary. There is frequent LOH involving 19p in ovarian and breast cancers (Osborne and Leech 1994; Jarrett, Blancato et al. 2001). In SW480 cell lines lacking functional APC protein, transfection with

the *APC2* gene inhibited beta-catenin mediated TCF signaling (van Es, Kirkpatrick et al. 1999).

Somatic mutations of *conductin* have been found in about a quarter of microsatellite-unstable cancers (Liu, Dong et al. 2000). All mutations were frameshifts in mononucleotide repeats and involved the disheveled and axin (DIX) domain, although bi-allelic mutations were not demonstrated. Conductin levels and mRNA levels have also been shown to be increased in human cancers and cancer cell lines with disrupted Wnt signaling and increased nuclear levels of beta-catenin. There is also an increase in levels in *APC* deficient Min mice. Beta-catenin stabilisation appeared to precede conductin up-regulation. Conductin may therefore be a target of the Wnt pathway, up-regulated in a negative feedback loop to control Wnt signaling activity.

The TGFβ signaling pathway through a cascade of SMAD proteins leading to effects within the cell nucleus is also known to be disrupted in germline by mutations in *SMAD4* on chromosome 18q21.1 giving rise to the Juvenile Polyposis syndrome (JPS) in about 30-50% of families (Howe, Roth et al. 1998; Woodford-Richens, Rowan et al. 2001). LOH at 18q is frequent (about 70%) in sporadic colorectal cancers and *SMAD4* mutations have been found in 16% of colon cancers (Thiagalingam, Lengauer et al. 1996; Riggins, Kinzler et al. 1997; Zhou, Buckhaults et al. 1998). The SMAD4-/+ mouse develops gastric and intestinal juvenile polyps and gastric cancer after several months (although not colon cancer) (Xu, Brodie et al. 2000). It acts as a tumour suppressor gene both in sporadic colorectal and pancreatic cancer and in polyps and cancer , from JPS patients. The polyps observed in JPS and the invasive cancers in the SMAD4+/- mouse show allelic loss, supporting the notion that bi-allelic inactivation of SMAD4 is needed for colon cancer formation (Woodford-Richens, Williamson et al. 2000). *TGFβ-II* is also frequently mutated in MSH-H tumours both familial and sporadic indicating a role in tumourigenesis or progression (Lu, Kawabata et al. 1998; Roth, Johansson et al. 2000). The TGFβ superfamily includes the BMP'S (Bone Morphogenetic Proteins). Germline mutations in *BMPR1A* have recently also been shown to cause about 38% of cases of JPS without *SMAD4* mutations (Zhou, Woodford-Richens et al. 2001; Sayed, Ahmed et al. 2002; Howe, Sayed et al. 2004). The mutations were predominantly nonsense leading to a truncated protein and loss of the wild-type allele was seen in these tumours. As with the TGFβ receptor, the best understood post-

BMP receptor pathway is the SMAD pathway including SMAD4 (Kawabata, Inoue et al. 1998).

Although none of the patient group tested was known to have developed juvenile polyps, these genes remain good candidates given their role in the development of colorectal cancer in both sporadic and hereditary forms. Some patients with juvenile polyposis syndrome – caused by germline *SMAD4/DPC4* or *ALK3/BMPR1A* mutations – also develop adenomas, although all cases described so far have had a personal or family history of juvenile polyps in addition to their adenomas.

Both *beta-catenin* and *axin 1* had been screened for in the germline of a similar set of patients so the candidate genes screened were *GSK3β, Conductin, APC2, SMAD4* and *BMPR1A.*

3.2 Methods

Screening of *Conductin/Axin2, APC2, GSK3β* and MSI testing were undertaken equally by Dr Oliver Sieber and I.

3.2.1 Selection of Probands

Probands were affected persons from 95 different families recruited through the Cancer Research UK Family Cancer Clinic at St Marks Hospital London and the Guys Hospital Clinical Genetics Department London. Forty-one were undergoing regular colonoscopy at St Mark's Hospital. All patients gave consent to the use of their DNA in a research context. The clinical features of the initial cohort of 47 are included in Table 3.1. Probands had developed between 5 and 115 adenomatous polyps in their lifetimes (synchronous or metachronous). Although 25% of patients had developed hyperplastic as well as adenomatous polyps, no patient had predominantly hyperplastic polyps. The majority (83%) had other family members affected with multiple adenomas and/or cancer. The relatively low incidence of cancer in this group reflects this as most had their first screening colonoscopy as a result of family history and from then on received regular screening colonoscopy, reducing the risk of colorectal cancer development. In all patients pathogenic germline mutations in *APC* were excluded using SSCP, DGGE and PTT. The majority did not undergo testing for large deletions and *APC* as a previous study had shown no such

mutations in a cohort of 100 multiple adenoma patients (Sieber, Lamlum et al. 2002),. All had *MYH* mutations excluded using SSCP and, some cases sequencing. Where cancer tissue was available (26 cases), microsatellite instability was excluded using the microsatellite markers BAT 26, TGFβRII and D5S346. As multiple colorectal adenomas are not a feature of HNPCC it was thought highly unlikely that MMR gene mutations could account for disease in the other 11 cancer cases.

3.2.2 Screening *APC2*

Primer pairs to amplify overlapping fragments were designed for the coding regions including exon-intron boundaries of *APC2* (exon one to codon 1388 in exon 14-including the "twenty amino acid repeat' region) (Table 3.3). Fluorescent SSCP (F-SSCP) was performed on PCR products. Fragments showing aberrant migration were re-amplified, and then sequenced in both forward and reverse orientations. The protein truncation test (PTT) was used for the remainder of exon 14 (see chapter 2 for method).

3.2.3 Screening *GSK3β*

Primer pairs to amplify overlapping fragments including intron/exon boundaries were used to amplify the 9 exons of *GSK3β* and F-SSCP run on the ABI 3100 at 18°C and 24°C (Table 3.4).

3.2.4 Screening *Conductin/axin2*

Primer pairs were designed to cover the entire coding sequence plus intron/exon boundaries of *conductin/axin2* (Table 3.5). The entire conductin sequence was not available on a public database and was therefore derived by performing a BLAST search of the cDNA against the HTGS sequence database. SSCP was performed on the Phast system. Aberrant bands were sequenced directly.

3.2.5 Screening *SMAD4* and *BMPR1A*

PRC-based germline *SMAD4* and *BMPR1A* mutation detection and LOH was performed in collaboration with Dr Kelly Woodford-Richens. PCRs were performed using the primers

detailed in Tables 3.6 and 3.7 which cover all exons and exon/intron boundaries of *SMAD4* and *BMPR1A*. PCR fragments were then subjected to SSCP (Phast system). Samples with aberrant bands were re-amplified and sequenced. For LOH analysis, DNA extracted from tumour tissue was amplified alongside the blood DNA from the patient using three microsatellite markers, D10S573, ALK3CA and ALK3GGAA. Products were run on the ABI377 and results were analysed using Genotyper software, with areas under the peaks (including stutter bands) compared for all informative (heterozygous) markers. Allelic loss was considered present if the relative ratio of normal:tumour peak was less than 0.5, or greater than two, thereby allowing for contaminating normal tissue within the microdissected tumour.

3.3 Results

3.3.1 *APC2*

Two silent (nt 687 G→A and nt 1317C→T) and two novel missense (nt 419 T→C; F140S and nt 967 G→C; G322R) mutations were found in the 5' region of *APC2*, which comprises the majority of the known beta-catenin binding/regulation domains (Table 3.2). The F140S variant in exon 4 occurred at an amino acid that is not evolutionarily conserved and does not create or abolish a splice site, so doubt must therefore be cast on its significance. The G322R missense variant was excluded as disease-causing due to its absence in other affected family members. Due to technical difficulties we were unable to amplify the last third of exon 14. This portion of the gene has the least (15%) homology to the *APC* gene; hence it is unlikely that mutations here would affect β-catenin binding or the function of the Wnt signaling pathway.

3.3.2 *GSK3β*

No germline variants, pathogenic or polymorphic, were detected in any of the 47 patients screened consistent with previous studies failing to detect pathogenic somatic mutations in colorectal adenomas/carcinomas (Sparks, Morin et al. 1998).

3.3.3 *Conductin/Axin2*

Two conductin germline variants wee seen in our study population. One polymorphism in intron six (IVS6+19G→T) was present in 8/47 (17.0%) of patients and one silent variant in exon6 (nt.635 C→T) was present in 3/47 (6.3%) of patients. No clearly pathogenic changes were found.

3.3.4 *SMAD4* and *BMPR1A*

Screening of the TGF-beta superfamily members, *BMPR1A* and *SMAD4* identified a single putative pathogenic change in *BMPR1A*, a three base-pair deletion at codon 360 resulting in the loss of a histidine residue. This amino acid is highly conserved and lies within the kinase domain of the protein, which is essential for the activation of the downstream targets SMAD1 and SMAD5 (Massague and Wotton 2000). In accordance with a pathogenic effect, LOH at the *BMPR1A* locus was detected in an adenocarcinoma from the patient (Fig 1.). The patient had no juvenile polyps and was diagnosed at the age of 58 with eight tubular adenomas of mild to moderate dysplasia, distributed throughout the colon. He had no family history of colorectal cancers or polyposis. Besides this alteration, two intronic polymorphisms (IVS6-26t/a and IVS11 – 11 T→C), two silent point mutations (nt 435 G→A and nt 777 G→A) and one previously reported missense polymorphism (nt 4 C→A; T2P) were detected in *BMPR1A* (Howe, 2001). No pathogenic sequence changes or polymorphisms were detected in the *SMAD4* gene. To further clarify the involvement of *BMPR1A* mutations in causing a 'multiple' adenoma phenotype, a second series of 48 *APC* and *MYH* mutation-negative patients was screened for *SMAD4* and *BMPR1A* changes, but no additional pathogenic variants were identified.

3.4 Discussion and Conclusions

No pathogenic mutations were found in the Wnt pathway components *conductin*, *GSK3β* and *APC2* in the germline DNA of patients with a multiple colorectal adenoma phenotype and without *APC* germline variants. This is despite our understanding of the Wnt pathway's central role in the pathogenesis of many colorectal cancers. Lack of degradation of beta-catenin results in its increased translocation to the cell nucleus leading to up-regulation of transcription of a number of genes involved in proliferation and growth within the colonic epithelium. Why then is *APC* the only piece of the pathway which is inactivated by mutations in the germline leading, ultimately, to the FAP and MA phenotypes?

One explanation may be our limited knowledge of the roles of APC and other Wnt pathway components within the cell. It may be that the functions of APC outside the Wnt pathway are the crucial ones for preventing adenoma formation, possibly those involving chromosome segregation, spindle orientation and microtubule integrity, mediated by the –COOH terminal of the gene. Since the *APC2* gene appears to have a high homology with *APC* except for the COOH terminal region, mutations in *APC* but not in *APC2* may be detrimental to the cell. The putative role for APC in maintaining chromosomal stability within the cell may not be shared by APC2. It also remains possible that the role of APC2 may be so great that a pathogenic germline mutation is lethal or that it is in truth a functionally redundant gene in the human.

GSK3β showed no variation at all in the 47 patients examined. No polymorphisms were seen. There is a possibility that mutations or allelic differences are not tolerated well and that truncating mutations may not be compatible with life. The findings are consistent with previous studies failing to detect pathogenic somatic mutations in colorectal adenomas/carcinomas (Sparks, Morin et al. 1998).

As previous studies have excluded germline *beta-catenin* and *axin 1* variants in a similar population of multiple adenoma patients, the analysis of genes whose products comprise the Wnt pathway provides little evidence implicating their involvement in this condition. This finding is supported by previous studies excluding germline beta-catenin variants in 'multiple' adenoma patients (Albuquerque, Cravo et al. 2002). This finding contrasts with that in the somatic mutation of spectrum of sporadic cancer. A large proportion of colorectal and other tumours without inactivating *APC* mutations are found to have activating somatic *beta-catenin* variants (Samowitz, Powers et al. 1999; Shitoh, Furukawa et al. 2001; Albuquerque, Breukel et al. 2002). *Beta-catenin* activating mutations may well be lethal in the germline.

The histology of the polyps in this group of mutation-negative 'multiple' adenoma patients did not differ in any obvious way from that of sporadic or FAP adenomas and thus gives no clue as to a specific genetic aetiology. One interesting feature of this cohort was the number of affected persons who appeared to have no dominant family history of colorectal cancer or polyps (57%). The one individual who was found to have an apparently pathogenic mutation

in *BMPR1A* had no juvenile polyps reported. He was diagnosed at the age of 58 with eight tubular adenomas of mild to moderate dysplasia distributed throughout the colon and had no significant family history. This may indicate that this gene contributes, albeit rarely, to a subset of patients who present only with multiple adenomas in a seemingly sporadic way. The absence of mutations in genes involved in the Wnt and TGF-beta signaling pathways suggests that other, as yet unidentified, predisposition genes and pathways exist. Possible candidates include the CRAC1 locus on chromosome 15q14-q22 (Jaeger, Woodford-Richens et al. 2003).

In conclusion, the 'multiple' adenoma phenotype cannot generally be attributed to germ-line mutations in genes involved in the Wnt and TGF-beta signaling pathways. Patients and families who present in this manner could be offered mutation analysis of the *APC* (AAPC-associated mutations), *MYH*, and perhaps *BMPR1A* genes, although the number of pathogenic mutations identified in the last of these will be relatively small. It is probable that a larger proportion of these patients (10-15%) will be attributable to *MYH* mutations and a smaller number due to attenuated *APC* variants. Further work is needed to unravel the genetic aetiology of multiple colorectal adenomas, but given the heterogeneity of the phenotype, this is likely to require a combination of genetic linkage analysis, examination of candidate genes in alternative pathways leading to adenoma formation and examination of tumours and adenomas from such patients to establish type and pathways of somatic mutations.

Table 3.1 Clinical Characteristics of Multiple Adenoma Patients.

Patient characteristics	**Number of patients**	**Mean**	**Median**	**Range**
Sex M:F	24:23			
Age of diagnosis		48.5	50	18-72
Family history	39/47 (83%)			
Dominant family history	27/47			
Adenomas	47/47 (100%)			
Adenoma count		21.8	12	3-100
No. with >15 adenomas	18/47			
Hyperplastic polyps	12/47 (25%)			
Colorectal cancer	9/47 (19%)			

Table 3.2 APC2 Germline Variants

Patients	Gene/exon	Mutation	Predicted effect of mutation
D1	APC2 exon 4	nt. 419 T->C	F130S
E6	APC2 exon 6	nt 687 G->A	silent
A1	APC2 exon 8A	nt 967, G->C	G323R
D3, H6	APC2 exon 10	nt. 1317 C->T	silent

Table 3.3 Oligonucleotide Sequences and PCR Conditions for APC2 Primer Pairs.

Exon	Sequence	Product size (bp)	MGC1 (mM)	Annealing Temp (C)
1	accatcagctgaaccctctg tagccacctcctccatga	220	1.0	55
3	ggagcagctgaagggtga gcgaattagagggcagagc	365	1.0	55
4	gccatgttgcctgttaacct atcgagaactgctggcaaag	361	1.0	55
5	cactcagggtgcgggaag ccgcccataatatctgcact	285	1.5	60
6	gggtcagctccagcacttg cgggcagaactcgaggag	193	1.0	60
7	tgggtgctctgggactgta acctgatccccccttccag	205	2.0	55
8A	gaccgggtttccaggtgt ctgcgagaagacgatgttgt	323	2.0	55
9	gtcacctgggacatttcctg agaggacctgggacactcac	172	2.0	60
10	accttgttgggtcctcacag tccctggaagactggatgag	213	1.0	55
11 + 12	gctgcataacccccaacag ccagtgactgaagaagcatcc	436	1.0	60
13	gtgagcgtgggagccttt cattcacgggaggacagtg	369	2.0	55
14 (PTTA)	ggatcctaatacgactcactataggaacag accaccatgctccgggatcacaactgtct	1699 + 42	1.5	55*
14 (PTTB)	ggatcctaatacgactcactataggaacag accaccatggagaactacgtgcaggagacac	1721 + 42		55*
14 (PTTC)	ggatcctaatacgactcactataggaacag accaccatgaattgtcacgtggctgcac	1837 + 42		55*

Table 3.4 Oligonucleotide Sequences and PCR Conditions for GSK3β Primer Pairs.

Exon	Sequence	Size (bp)	MgCl (mM)	Annealing Temp.
1	aaaggtgattcgcgaagaga tattttaagggcgaggtgga	164	2.0	55
2	ttcctacagttctcgtgaaaaa tgtattacggcagcacaaaaa	284	3.0	50
3	tgttatttcagaagcatcttgttca ttatgagcggtggggagatta	245	2.0	60
4	aagaggctctccttggttca gaccagttctaatctgtttgtttt	228	1.0	55
5	ctttgatactgtgaaaggatagca aaattaccaaaatcaagaagca	259	3.0	55
6	caaacacttttcagctcaagctat gcacaaaaattccttccagttc	206	1.0	60
7	cagtgaatccaatgcctgaa catgcacaactgcttgtattcat	293	1.0	55
8	atgtttctgtcacatggtgggaat acccagggtgtagctttcct	343	2.0	55
9	gcccggcataaactggtagt gctgctgtggcatttgtg	209	1.0	60

Table 3.5 Oligonucleotide sequences and PCR conditions for Axin2 primer pairs.

Exon	Sequence	Size (bp)	MgC1 (mM)	Annealing T
1A	ccagactcagtgggaagagc cagaggggaatccggagat	254	2	
1B	cggaacgaagatgggttg tgcttcttgatgccatctctt	323	2	55
1C	cctgccccaccaagacctacat aagtcggcacaagtccactc	266	2	60
1D	ctccccaccttgaatgaaga tctgcaaagtccacagcatc	191	1	55
2	ggtttctggtgagggtccagt tctggctaactgctcaggtg	258	1	60
3	cccatgtgatgtgctgaaaa tcaacatggcagaaaacagc	213	2	60
4	ctcctccctttcctttccac gacggaagcaggaagaagg	204	1	60
5A	ccttctgacgtcttccctttc ggagtggtactgcgaatggt	260	1	55
5B	gccagtctccaggcgtag ggcatggtggtggatgtagt	215	1	55
5C	ccttgtgaccaagcagacga ggcacttacccaaactgctc	210	1	60
5D	aatgcaaaagccactccaag gctggtgaccacgaaagacc	225	1	55
5E	ttctaagagggcggcagag tgcgacctgtctccttcc	300	1	60
5F	caaacgcaatgggaaagg cagccaattcccacaatacct	245	2	55
6	tctgtttctctctgctcattcc gcctcaacctaggacccttc	297	1	60
7X	aattctctggggacaacag agcactcggcagatctcagt	195	2	60
7Y	tgctaaacttgttccattcca tctggctcttggttctgagc	260	2	60
7Z	cctcagtcctccatgttggt cagttcaccaaagccagacc	241	2	60

Table 3.6 Oligonucleotide Sequences for SMAD4 Primer Pairs.

Exon	Sequence	Product Size (bp)
1	ttgcttcagaaattggagaca gcttgaaaggaaacgtagcaa	385
2	tgtatgacatggccaagttag caatactcggtttagcagtc	530
3	ctgaattgaaatggttcatgaac gcccctaacctcaaaatctac	308
4	ttttgctggtaaagtagtatgc ctatgaaagatagtacagttac	509
5 + 6	catctttatagttgtgcattatc taatgaaacaaaatcacaggatg	557
7	tgaaagttttagcattagacaac tgtactcatctgagaagtgac	224
8	ggatgttctttcccatttat acaatcaataccttgctctc	224
9	tattaagcatgctatacaatctg cttccacccagatttcaattc	332
10	aggcattggttttaatgtatg ctgctcaaagaaactaatcaac	293
11	ccaaaagtgtgcagcttgttg attgtattttgtagtccacc	570

Table 3.7 Oligonucleotide Sequences for BMPR1A Primer Pairs.

Exon	Sequence
1	tccaaaattcagttgtattcc cacatacattactaaaatgaacactg
2	gtcacgaaacaaatgagcttt ttaagaagggctgcataaaa
3	cattcagactcaaatttcgtt tctcatgggtcccaaatta
4	ccaaaccatttctaattttatca catgctccgacttttctc
5	ccaggctacctagaattgaa aacagcggttgacatctaat
6	cctcaggttttcttaggg tcaacacaccaattcatgtct
7	tcatcaagagctcaaacctt acctcactagccttgtcaaa
8	ccctagcctatctctgatga aacagtggggcaaagaac
9	tattttattttggccctca tgatgagtaaatcaacataatcag
10	atttttgtgcccatgtttt aatcacttcttcaggggact
11	actcagtcccctgaagaagt ctagagtttctcctccgatg

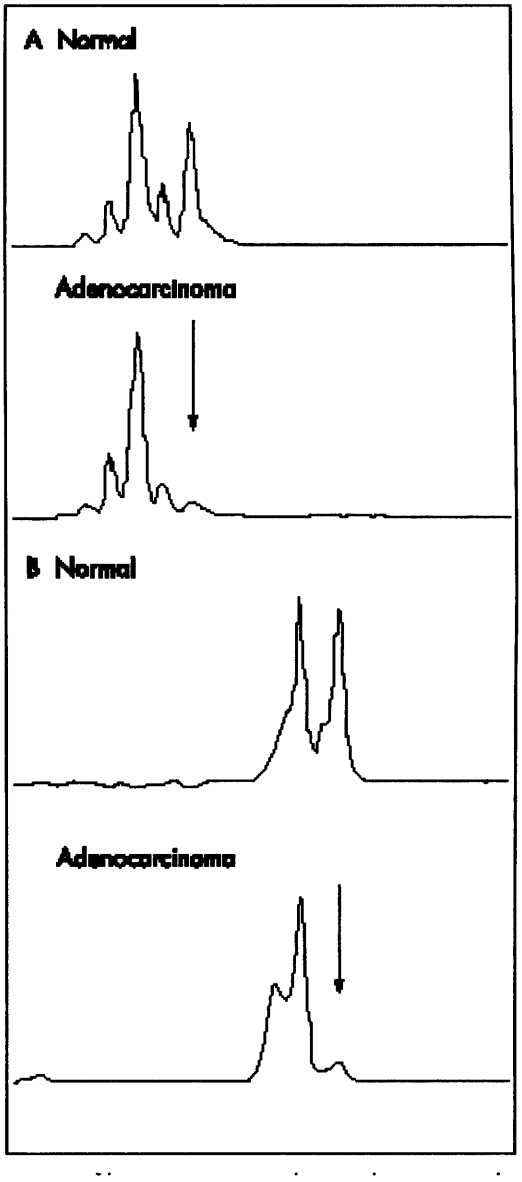

Figure 3. 1 Loss of heterozygosity analysis

LOH at the BMPRIA locus in an adenocarcinoma from a patient with a germline *BMPRIA* mutation. Shown are the results from microsatellite (A) ALK3CA and (B) ALK3GGAA lying 43 Mb and 73Mb proximal to the BMPRIA gene, respectively. The allele showing LOH is arrowed.

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Chapter 4 The Multiple Colorectal Adenoma Phenotype, Familial Adenomatous Polyposis and Germline Mutations in *MYH*

4.1 Introduction

Patients with five to about one hundred colorectal polyps are very heterogeneous group. Although *APC* mutations account for a proportion of individuals who develop such a multiple adenoma (MA) phenotype, the majority remain without genetic diagnosis at this time. Dominant and recessive family histories, early and late ages of onset and different prevalence of hyperplastic and adenomatous polyps all exist within this group. One of the main aims of this work has been to account for cause of polyposis in some of these individuals and families.

Amongst those individuals diagnosed with FAP on the basis of adenoma numbers (>100) and characteristic histology about 30% do not have a detectable germline *APC* mutation. Although some of this group can be accounted for by large genomic deletions and cryptic *APC* mutations a significant number remain without known genetic cause (Lamlum, Al Tassan et al. 2000). The phenotypes of persons diagnosed with FAP or MA on clinical grounds has much overlap. Extra-colonic features and autosomal dominant inheritance of polyposis are the major features to suggest the likelihood of an APC mutation as the cause of disease.

A further distinct group of patients is known to develop predominantly hyperplastic polyps. Although it is common to have a few small hyperplastic polyps in the distal colon or rectum, a group of patients develop, large, multiple and/or proximal hyperplastic lesions and are at increased risk of colorectal cancer (Whitelaw, Murday et al. 1997; Rashid, Houlihan et al. 2000; Hyman, Anderson et al. 2004). The syndrome of hyperplastic polyposis does not appear to have a dominant inheritance and few such families have been described. Most cases are isolated or recessive and present at all ages.

The previously described candidate gene analysis (Chapter 3) was the first step taken to try to account for the MA presentation in some of these individuals. In February 2002 Al-Tassan *et al* described a family in which three siblings developed multiple colorectal adenomas and cancer (Al-Tassan, Chmiel et al. 2002). They did not have a germline mutation in *APC*

detected. Whilst screening tumour samples form these patients for somatic changes in *APC*, including the E1317Q missense change it was noted that there appeared to be an excess of somatic G→T transversion mutations compared with the normal somatic *APC* mutation spectrum. Since oxidative damage is a major cause of G:C→T:A mutations, genes responsible for the repair of such damage were then examined for mutations in the germline of these siblings.

8-oxo-7, 8-dihydro2'deoxyguanosine (8-oxodG) is the most stable and mutagenic product of oxidative DNA damage. It readily mispairs with A residues leading to G:C→T:A mutations in repair deficient bacteria and yeast (Olinski R 1992; Okamoto K 1994). The products of three human base excision repair genes, *MTH1*, *OGG1* and *MYH*, act synergistically to prevent 8-oxo-dG induced mutagenesis. In the nucleotide pool, the human MutT homologue, *MTH1* hydrolyses 8-oxo-dGTP to 8-oxo-dGMP, preventing incorporation of the oxidised G nucleotide during DNA replication (Nakabeppu 2001). The human MutM homologue, *OGG1* detects and removes 8-oxoG adducts that have been mis-incorporated into the DNA opposite C residues leaving a single strand gap (Slupska, Baikalov et al. 1996). The human MutH homologue, *MYH*, an adenine-specific DNA glycosylase, scans the daughter strand after replication, removing adenines mispaired with 8-oxo-dG or guanines (Slupska M.M 1999; Lu, Li et al. 2001; Gu, Parker et al. 2002)(Fig. 4.1).

Al-Tassan found that the live affected members of the family were compound heterozygotes for the missense mutations Y165C and G382D in the human homologue of the bacterial MutY gene – *MYH*. These variants are in exons seven and 13 respectively. These positions are highly conserved in MutY across species.

Chmiel et al performed analysis of the corresponding mutations in E. coli Mut Y, Y82C and G253D (Chmiel 2003). This revealed a reduction in the adenine glycosylase activity of the enzymes, especially Y82C. In vivo complementation assay using the mutant human MYH alleles in Mut Y E.coli cells showed that complementation by these alleles was possible and that the mutations significantly decreased enzyme activity. Y165C was markedly more deleterious in its effect on BER than was G382D.

We set out to establish whether these or other variants in the *MYH* gene existed in MA patients and in *APC* mutation negative FAP patients and, if so, in what proportion. Patients

found to be double mutation carriers had cancer and adenoma DNA examined for evidence of BER disruption in the form of an excess of G: C→T: A mutations in the *APC* gene. We also wished to fully describe the clinical condition and variants if any arising from such mutations. Lastly we wished to exclude any contribution from other genes involved in 8-oxodG repair in the MA phenotype and classical polyposis phenotype.

4.2 Methods

4.2.1 Patient Population

The patient group was recruited by Dr Ian Frayling and Dr Ella Barclay and I.

For the MA subgroup we identified 156 patients with multiple (five to 100) synchronous or metachronous colorectal adenomas from Genetics Departments in the United Kingdom (St Mark's Hospital, Harrow; Oxford; and Guy's Hospital, London) . Patients had been referred either owing to a family history of colorectal tumours or after they themselves had presented symptomatically with multiple polyps. All patients had consented to provide a blood/DNA sample and access to archival tissue specimens.

Clinicopathological data were ascertained from patients' records to confirm diagnoses. In all cases, either a precise adenoma count had been reported, or more rarely, a rounded or approximate count was given. Family histories (of tumours or other major disease) were recorded as reported by the patients and were confirmed, where possible, from hospital records, although precise adenoma counts in patients' relatives were rarely available. We used 107 anonymous, random United Kingdom controls, derived from tumour-free individuals from a national study unrelated to cancer. We also analysed germline DNA from 26 patients with multiple adenomas from Finland and Denmark, all of whom had been reported as having between 5 and 100 adenomas.

For the mutation-negative FAP subgroup we contacted polyposis registries in the United Kingdom, Switzerland, Finland, Portugal and Denmark with a request to study all *APC* mutation-negative patients with more than 100 adenomas (synchronous or metachronous). We ascertained 110 probands and confirmed that local laboratories had rigorously excluded germline *APC* changes. Full clinicopathological details and family history were obtained.

For some patients, exact polyp counts at colectomy had been recorded; for others, counts were given in a range (for example, "100-1000" or "several thousand" to distinguish mild and severe classical polyposis respectively); and for yet others, counts were provided essentially for diagnostic purposes (that is, >100).

A group of 42 patients with hyperplastic polyposis was ascertained from St Mark's Hospital, Harrow (Table 4.1). Information regarding their polyp number, size and histology was assessed such that all patients included in the analysis had either >10 polyps with more hyperplastic than adenomatous polyps or at least one large (>10mm) hyperplastic polyp.

4.2.2 Mutation Detection

All mutation detection was performed equally by Dr Oliver Sieber and I.

Germline DNA was extracted form lymphocytes (see chapter 2). F-SSCP with direct sequencing of aberrant bands was used for germline *MYH* (GenBank NM_012222), *hMTH* (AB025241), *OGGI* (NM_002542, NM_016821) mutation detection (see chapter 2). Primer pairs to amplify overlapping fragments were designed for the coding regions including exon-intron boundaries of the *MYH*, *hMTH* and *OGG1* genes (Tables 4.7-9). Patients found to have a single *MYH* mutation had direct sequencing of the entire MYH gene performed to rule out a second mutation. Tumour tissue was extracted from archival or fresh frozen specimens by standard methods (see chapter 2). *APC* mutation testing on tumour specimens was performed using F-SSCP and dHPLC. The standard *APC* primer set for exons 15A to 15L was used for analysis of fresh frozen tissue (Table 4.10). For paraffin-derived DNA, a set of overlapping primers was used to examine the APC gene from codon 1219 (exon 15F) to codon 1609 (exon 15J), the area incorporating the mutation cluster region (MCR) and where the majority of somatic *APC* mutations had been found by Al-Tassan's group in the previously described sibship.

4.2.3 Loss of Heterozygosity and Genotyping Analyses at D1S2677

Loss of heterozygosity (allelic loss) and genotyping analyses at the microsatellite D1S2677 (2.5 kb from *MYH*) were performed using standard protocols, dye-labeled oligonucleotides and the AB1377 sequencer. Allelic loss was scored if the dosage of one allele in the tumour

decreased by 50% or more relative to the other allele, after correcting for the relative allele peak areas using constitutional DNA.

4.3 Results

4.3.1 Germline *MYH* Mutations in Multiple Adenoma Patients

The 156 United Kingdom patients with multiple adenomas (Table 4.2) presented at median age 56 (range 16-77) years. To the date of follow-up (31st December 2001), the mean number of synchronous or metachronous adenomas developed throughout life was 16 (median 7, range 3-100). Twenty-eight patients presented with a synchronous colorectal carcinoma, but none was known to have developed a cancer subsequently, all patients having been placed on regular surveillance colonoscopy. A family history of colorectal cancer (with any inheritance pattern) was reported by 94 patients. Of these, 65 families displayed dominant inheritance with two generations only known to be affected. In 17 families more than two generations were affected and in 12 families inheritance was compatible with autosomal recessive inheritance. A further 52 individuals reported no family history of colorectal cancers or adenomas. No patient had a family history of adenomas without also having a family history of colorectal cancer. No patient reported consanguineous origins.

Ten patients carried bi-allelic *MYH* mutations (Table 4.2). Of these, six were compound heterozygotes, as shown by sequencing of cloned PRC products; the remaining four were presumed homozygotes. The previously reported missense changes, Y165C (494 A>G) and G382D (11145 G>A), were the most common alterations representing 16/20 (80%) of all mutant alleles in biallelic mutation carriers. Both Y165C and G382D target highly conserved residues, the former mapping to the helix-hairpin-helix protein domain which confers specificity of mismatch recognition and the latter affecting the catalytic core of the glycosylase (residues 366-497). The novel frameshift changes, 1103delC (codon 368) in patient IV and 1419delC (codon 473) in patient III were also identified which are predicted to abolish glycosylase function. The sister of III was also diagnosed with two synchronous cancers and multiple adenomas at the age of 71 and had the same two mutations in *MYH*. Patient IX and his brother were affected with rectal cancer and multiple adenomas in their thirties and were both homozygotes for the nonsense mutation E466X (1396 G>T). This variant in exon 14 of *MYH* has since been found to occur primarily in persons of Indian

extraction (Jones, Emmerson et al. 2002). In the youngest double mutation carrier adenomas developed at 15 years of age and he died of metastatic gastric cancer at age 16 (Patient VII).

None of 107 United Kingdom controls carried two *MYH* mutations. Y165C was found in just two controls and none of the other disease-associated mutations was present in the control group. The previously described *MYH* polymorphisms in exon 2 (64 G>A;V22M), exon 12 (972 G>C; Q324H) and exon 16 (1502 C>T; S501F) were detected with respective allele frequencies of 10 percent, 21 percent and 2 percent in our patients, similar to the control population previously reported (Al-Tassan, Chmiel et al. 2002).

In order to provide further evidence of the pathogenicity of the *MYH* mutations, available relatives were screened for the changes carried by the proband (Table 4.3). In all cases, the results were consistent with recessive inheritance. For patient I, an affected sister was a mutant homozygote. For patient II, an unaffected daughter was a heterozygote. For patient III, her sister was affected as stated. For patient VI, only unaffected siblings survived and they were both heterozygotes. For patient IX, an affected brother was a mutant homozygote (Figure 4.1).

All but one (patient IX) of the United Kingdom patients with bi-allelic *MYH* mutations was of northern European ancestry. In order to determine the frequency of Y165C and G382D in different populations, 26 Finish and Danish individuals with multiple adenomas were investigated. Two (8%) of the patients were compound heterozygotes for Y165C and G382C. In the entire patient set, Y165C and G382C were not consistently associated with specific alleles at D1S2667, thus providing no evidence for these being ancestral rather than recurrent changes.

Six patients were heterozygous for an *MYH* mutation and the wild type allele (Table 4.3). In these individuals, we sequenced the entire *MYH* gene, but found no further changes. We screened seven adenomas from one of the G382D carriers for somatic *APC* mutations and found two changes, one C>G transversion (S1346X) and one 1bp deletion (4244 delG). Neither change involved G>T transversion and hence was not suggestive of defective MYH activity. A further screen of *APC* exons 15 A to L in five patients with colorectal cancer and heterozygous for Y165C (one) or G382D (four) revealed one G>T mutation (E1378X) in the Y165C carrier.

4.3.2 *MTH1* and *OGG1* Screening in Multiple Adenoma Patients

No carriers of obviously pathogenic or bi-allelic *MTH1* or *OGG1* mutations were detected in 127 and 42 multiple adenoma patients respectively. For *MTH1*, the allele frequency of the previously described C>T codon 160 polymorphism was not significantly different from previously described controls accounting for 18% of alleles in our cohort. In addition, a novel *MTH1* missense variant (92 G>A;R31Q) was identified in one patient, but not in the controls, but this mutation did not co-segregate with the multiple adenoma phenotype. For OGG1, besides the well-described polymorphism in exon 7 which accounted for 27% of alleles (977 C>G;S326C) no further DNA alteration were detected (Kohno, Shinmura et al. 1998), (Wikman, Katballe et al. 2000; Hanaoka, Sugimura et al. 2001) .

4.3.3 Genotype-phenotype Associations in Multiple Adenoma Patients

Eight of the ten patients with bi-allelic *MYH* mutations presented at a similar age (median 56 years, range 45-59) to the other multiple adenoma patients in our study (Tables 4.2 and 4.5). Two presented at younger ages (16 and 34 years). Nine individuals had symptoms at presentation and one was found to have polyps at colonoscopy performed owing to a family history of colorectal tumours. Polyps were predominantly small, mildly dysplastic tubular adenomas, with a minority of tubulovillous adenomas and very few hyperplastic polyps. Five of the ten patients had colorectal cancer at presentation. Seven of the ten had a family history of colorectal cancer, in five cases involving more than one generation. Carries of bi-allelic *MYH* mutations were no more likely to have a family history of colorectal cancer than other patients in the study (p=0.10, Fisher's exact test). Four *MYH* patients had confirmed family histories of multiple adenomas, but these were found only in siblings, consistent with recessive inheritance of MYH-associated disease. One extra-colonic tumour, a gastric cancer, was reported. No other clinical features were reported.

The one phenotypic difference which clearly distinguished patients with bi-allelic *MYH* mutations from single mutation carriers and *MYH* mutation-negative individuals in our multiple adenoma patient set was the number of tumours (medians of 40 vs. 4 vs. 7 respectively; p=0.023, Kruskal-Wallis test) (Table 4.2). Of 27 patients with 15 to 100 adenomas, ten (38.4 percent) harboured bi-allelic *MYH* mutations.

The colorectal cancer frequency (6 out of 10) in bi-allelic *MYH* mutations carriers was greater than that in the other patients in our study (p=0.05; Fisher's exact test) as well as the general population (3.86 percent for 0 to 80 years of age; http://seer.cancer.gov/). These data suggest an increase risk of bowel cancer in bi-allelic *MYH* mutants, although this result should be interpreted with much caution, because, whilst these patients wee recruited on the basis of their adenoma phenotype alone, they were more likely to have come to clinical attention if they had a synchronous carcinoma.

Following the study of this group of multiple adenomas patients, two further bi-allelic *MYH* carriers were found on screening multiple adenoma probands. The first patient, a compound heterozygote for Y165C/G382D was, diagnosed at 68 with colorectal cancer and had two siblings diagnosed with colorectal cancer at 46 and 61. There was no record of adenomas being present in this family. The second patient was male and diagnosed at 50 with a Stage I carcinoma of the rectum and 25 adenomas. He had one sister diagnosed with CRC at 54 years and one brother who had a few small adenomas at the age of 56 years.

4.3.4 *MYH* Mutation Testing in Patients with Classical FAP

Eight of 107 probands with classic polyposis (7.5 percent) carried biallelic pathogenic *MYH* mutations (Tables 4.4 and 4.5). Y165C and G382D were again the most common changes. Three other mutations were found: a frame shift (252delG at codon 84); an unusual in-frame duplication (411dupATGGAT at codon 137, 137insIW); and a non-conservative missense change (G->T at position 694, V232F). Four patients carried single *MYH* mutations (Y165C in two patients, I209V in one, and G382D in one).

All probands with polyposis who had biallelic *MYH* mutations had a family history compatible with recessive inheritance, in that only the proband or siblings in a single generation were affected by polyposis. Although it is necessary to exercise some caution, given the variation among centers in clinical practice and in the precision of the counting of polyps, it is probable that patients with *MYH* mutations had mild classic adenomatous polyposis: none had more than 1000 polyps; two had exact counts of 115 and 210 adenomas; and none had early-onset cancer. All patients with two *MYH* mutations had been treated by

total colectomy with ileorectal anastomosis or ileal pouch, at a mean age of 47.6 years (median, 47; range, 30 to 70), as compared with a mean of 28 years (median, 23; range, 13 to 65) among patients with *APC* mutations who were included in the polyposis registry of St Mark's Hospital (data not shown) (1998).

In several respects, the clinicopathological features of patients with biallelic *MYH* mutations were the same as those of patients with polyposis resulting from *APC* mutations: macroadenoma morphologic features were the same (largely small tubular lesions with mild dysplasia); microadenomas were present, despite the fact that such lesions were previously thought to be pathognomonic of classic adenomatous polyposis; and some patients had extracolonic disease. Severe (Spigelman stage IV) duodenal polyposis developed in Patient 15, and Patient 16 had duodenal polyps at diagnosis. Congenital hypertrophy of the retinal pigment epithelium was diagnosed in Patient 13 (although it was not specifically noted to be of a type associated with polyposis). No desmoid tumors were reported.

4.3.5 *MYH* Testing in Patients with Hyperplastic Polyposis

42 patients with hyperplastic polyposis were tested for variants in *MYH* (Table 4.1). The mean age of diagnosis was 54 (median 58, range 19-72 years). The exact polyp number was available for 34 patients with others being recorded as 'multiple'. Mean number of polyps where available was 44 (median 35, range 6 to 100). Overall 16/39 patients (41%) were found to have only hyperplastic polyps whereas the others also developed adenomatous polyps including tubular, tubulovillous, villous and serrated adenomas. 23/37 patients (62%) had right sided polyps and 21/36 patients (58%) had hyperplastic polyps $\geq$1cm diameter). Family history of colorectal cancer or polyps was reported in only 10/42 (23%) of cases, some of which were not first degree relatives. No patient was found to carry any pathologic *MYH* variant.

4.3.6 Somatic *APC* Mutations in Adenomas and Cancers from *MYH* Double Mutants.

115 adenomas and 8 cancers from 15 *MYH* bi-allelic mutations carriers (table 4.5) were screened for somatic *APC* mutations from codon 1219 (exon 15F) to codon 1609 (exon 15J). Five further cancers from five patients were screened for exons 15A to 15L. Six out of 14

(43%) of cancers and 22/105 (21%) of adenomas showed somatic *APC* mutations. Details of somatic *APC* mutations are shown in table 4.6. All are G:C>T:A transversion mutations.

Loss of heterozygosity at *MYH* was found in seven of 62 informative adenomas from the *MYH* mutant compound heterozygote patients. Given that G382D reportedly retains some enzyme activity, the low frequency of allelic loss may indicate that absent MYH function is not necessary for tumourigenesis (Chmiel 2003).

4.4 Discussion

Conclusions drawn from the study were made by me, Dr Oliver Sieber and Professor Ian Tomlinson.

Germline *MYH* mutations, both missense and truncating, predispose to apparently recessive inheritance of multiple colorectal adenomas and classical adenomatous polyposis in both European Caucasian and Asian populations. All patients with bi-allelic *MYH* mutations probably have raised risk of colorectal cancer. Of patients with 3-100 adenomas, about 5% had disease ascribable to bi-allelic *MYH* mutations, and of those with over 15 adenomas, over one third had bi-allelic *MYH* mutations. In patients with a phenotype of classical polyposis and no detectable *APC* mutation, about 8% of cases had two germline *MYH* mutations. Extra-colonic disease was present in some patients with *MYH*-associated polyposis, showing that these features are not restricted to those with germline *APC* mutations. The presence of extra-colonic disease is consistent with the model of mutant MYH action in the colon, namely hypermutability of *APC* (and possibly of other genes also). This is borne out by the frequency of somatic *APC* mutations seen in tumours from patients with MYH associated polyposis.

It appears from this study that patients with bi-allelic *MYH* mutations tend to have mild colonic disease relative to most patients with classical familial adenomatous polyposis, but more severe disease than most multiple adenoma patients. It is certainly difficult to distinguish between patients with *APC* and bi-allelic *MYH* mutations on the basis of clinicopathological features, although family history can be useful. *MYH* mutations appear to be a more frequent cause of the multiple adenoma (or 'attenuated polyposis') phenotype than are *APC* mutations, but are evidently a less frequent cause of classical adenomatous

polyposis (Lamlum, Al Tassan et al. 2000). Compared with hereditary non-polyposis colon cancer, carriers of bi-allelic *MYH* mutations develop more tumours, but taking the limited clinical data from this study, progression from adenoma to carcinoma appears to be slower. It remains difficult to comment on the tempo of disease development and progression in MYH associated polyposis for several reasons. Firstly, due to the recessive seeming nature of the condition, patients are diagnosed late and often require colectomy for multiple polyps at the time of diagnosis, preventing follow-up. In these persons it is impossible to say how quickly polyps developed. Secondly without large studies of populations undergoing mutation detection and colonic surveillance it is difficult to estimate the true risk to the biallelic mutation carrier and true average age of polyp onset. Thirdly if persons with MYH associated polyposis are detected at a time when polyp numbers are few because of screening programs they may continue to have small 'crops' of polyps removed and never present a 'polyposis' phenotype and hence not be drawn to the attention of family cancer clinics.

Six patients with one mutated *MYH* allele only were identified in the multiple adenoma cohort and four in the classical polyposis cohort. *MYH* mutant heterozygotes may have a weak susceptibility to bowel tumours, given that allelic loss on chromosome arm 1p is apparently an early event in colorectal tumourigenesis which could inactivate the wild type *MYH* allele (Tanaka, Yanoshita et al. 1993; Lothe, Andersen et al. 1995; Praml, Finke et al. 1995). In this patient set, there was no over-representation in general of heterozygotes as compared to our and published controls. In a recent publication however, a group of over 400 Finnish controls have been screened for the Y165C and G382D variants with neither being present (Enholm, Hienonen et al. 2003). The estimate of one to two percent of the population found in our study and that of Al-Tassan et al may be falsely high or may represent the British (Largely Caucasian) population only (Al-Tassan, Chmiel et al. 2002).

The two somatic *APC* mutations in polyps from an *MYH* heterozygote individuals were not G:C→T:A transversion mutations. Nevertheless, several of our patients had family histories suggesting dominant inheritance of colorectal cancer (although not of multiple adenomas). Formal exclusion of *MYH* as a weak susceptibility allele will require a large set of colorectal cancer cases and controls. This remains an important issue as regards screening of family members. If *MYH* mutations are present in 1-2% of the population at large it may indeed represent a low penetrance allele responsible for a not insignificant proportion of colorectal cancer.

Many questions remain regarding the specificity of *MYH* in causing colorectal adenomas and cancers. We cannot yet answer the question of why *MYH*, rather than *MTH1* or *OGG1*, is important for tumour predisposition and development. Specifically, we cannot exclude the possibility that carriers of bi-allelic *MTH1* or *OGG1* mutations are predisposed to tumours, although we found no such individuals in our patient set. Neither is it clear why the colon primarily appears to be affected rather than other tissues exposed to damage by reactive oxygen species such as the lung. It is entirely possible that we have missed an effect of *MYH* mutations in the genesis of other cancers in this study. We have targeted a group of persons with colorectal adenomas and cancers and often with a family history of such which has brought them to the attention of the family cancer clinic. Other cohorts of persons with different types of cancers and family histories need to be assessed to rule out an effect, particularly of the heterozygote state, on predisposition to other cancer types.

It does appear likely that bi-allelic *MYH* mutations do not account for the syndrome of hyperplastic polyposis. The sample used was well representative of such patients and most showed a sporadic/recessive pattern of disease with none having a dominant family history of polyposis, hyperplastic or other. These patients form a distinct and interesting group for further study.

Genetic testing for *MYH* mutations should be performed in patients with tens or hundreds of colorectal adenomas with or without colorectal cancer, subject to the proviso that almost all patients with MYH-associated polyposis will have a family history consistent only with recessive inheritance of multiple adenomas. Families may have an apparently dominant history of colorectal polyps because of a heterozygote effect or because of phenocopies in a disease common in the population. The study by Enholm et al and others by Wang, Fleischman and Zhou indicate that persons with under 10 adenomas may still be biallelic mutation carriers (Enholm, Hienonen et al. 2003; Fleischmann, Peto et al. 2004; Wang, Baudhuin et al. 2004; Zhou, Djureinovic et al. 2005). It seems probable that as population screening programs for colorectal cancer develop more patients with MYH associated polyposis will be seen at younger ages and with lower adenoma numbers. Screening of *APC* and *MYH* may be performed in parallel in some patients, such as isolated or recessive appearing cases of polyposis or those with tens to 100's of polyps. In a majority of cases with bi-allelic mutations one of the mutant alleles is Y165C or G382D, therefore it is a

simple matter to screen for these to changes first and then proceed to a whole gene screen if one mutation is found. In the Indian and Pakistani populations however, where there is evidence of common variants in exon 3 and 14 respectively these should also be screened. Evidently, if bi-allelic *MYH* mutations are identified in a proband, testing of siblings is worthwhile, even if they are asymptomatic in their 6^{th} or 7^{th} decade. It should, however, be borne in mind that in up to 2% of cases, a carrier of two *MYH* mutations will produce children with a spouse/partner who carries a single mutation; in this case the disease will appear to be dominantly inherited. The question remains open as to whether it is worthwhile to undertake genetic testing in the spouses/partners of MYH associated polyposis patients.

All but three of our patients with bi-allelic *MYH* mutations proceeded to colectomy, since their disease could not be controlled by colonoscopic polypectomy. For patients with relatively mild disease, regular screening by colonoscopy may be used initially – although optimal screening intervals must be determined empirically – and may prevent colectomy. In our cohort two patients with fewer than 50 adenomas and one with 100 could be managed by sequential colonoscopy and multiple polypectomies. Unfortunately, as mentioned, the recessive nature of the disease means that it will prove difficult to identify *MYH* mutation carriers at an early enough age to prevent colectomy in all cases. Management of single mutation carriers is currently unclear. Until population based studies are done it may be prudent not to abandon screening in these persons. Yet, in the absence of any evidence for an increased risk of colorectal tumours in *MYH* heterozygous mutation carriers, there is currently little justification for aggressive screening colonoscopy of any heterozygote family member. All individuals with two, identified *MYH* mutations probably should have regular endoscopy of the upper-gastrointestinal tract using a side-viewing endoscope, primarily for the detection and management of duodenal polyposis.

Molecular methods should now be used to classify disease in patients with multiple adenomas or adenomatous polyposis. Patients with identified germline mutation(s) should be classified as having *APC* or *MYH*-associated disease. Risks for relatives and the likely severity of disease can then be accurately assessed. Patients with polyposis but no identified germline mutation may then be further classified as presumed familial adenomatous polyposis if they have a dominant family history of classical disease and/or severe polyposis (>1000 colorectal adenomas). Other patients with no detected germline mutation in *APC* or

MYH, and with either mild polyposis (100-1000 adenomas) or multiple (<100) adenomas should be classed as having 'polyposis/multiple adenomas of unknown origin'.

Table 4.1 Patients Ascertained with Hyperplastic Polyposis.

HPP = hyperplastic polyp, TA = tubular adenoma, TC = transverse colon, HF = hepatic flexure, AC = ascending colon, DC = descending colon, SC = sigmoid colon, CM = caecum.

Patient	Age at Diagnosis	Total Polyp no.	HPP	TA	Polyp Site	Largest HPP (mm)	Colon Cancer	Colon Cancer Site	Family History CRC
1	55	53	yes	no	all colon	30	yes	TC	no
2	23	10	yes	no	right	16	yes	TC	no
3	28	multiple	yes	no	all colon		no		nF
4	60	multiple	JP			30			no
5	61	50	yes	no	left	5	no		no
6	37	100+	yes	yes	all colon	15	no		yes
7	38	multiple	yes	yes	left	8	yes		
8	67	50-100	yes	yes	all colon	8	no		yes
9	69	40+	yes	yes	all colon		no		yes
10	56	30+	yes	yes	TC on	5	no		yes
11	70	92	yes	yes	all colon	18	yesx2	HF+AC	no
12	64	25+	yes	yes	all colon	10	no		no
13	58	30	yes	no	left	5	no		
14	60	20	yes	no	all colon	7	no		no
15	19	multiple	yes	no	all colon	4	yes	AC	yes
16	65	35	yes	no	TC on	3	no		no
17	56	multiple	yes	no	left		no		yes
18	70	6	yes	no	left		no		no
19	72	30+	yes	yes	all colon	6	yesx3	SC,TC,HF	no
20	58	55	yes	yes	TC on	9	yes	TC	no
21	44	7	yes	no	all colon	10	no		yes
22	60	47	yes	no	TC on	5	no		no
23	71	70+	yes	yes	all colon	14	yes	SC	no
24	69	28	yes	yes	all colon	20	yes	caecum	
25	27	40	yes	no	all colon	10	yes		no

26	48	108	yes	yes	all colon	20	yes	CM	no
27	58	13	yes	yes	all colon	10	no		yes
28	26	50+	yes	yes	left	10	no		no
29	58	50-100	yes	yes	all colon	15			yes
30	63	multiple	yes	no	all colon	10	yes	TC	no
31	60	100+	yes	yes	right	12	yesx2	HF+DC	no
32	68	50-100	yes	no	all colon	40	no		yes
33	57	25	yes	yes		3	yes	HF	no
34	32	25+	yes	yes	left	10	no		
35	61	50+	yes	yes		12	yes	CM	no
36	54	?	yes	no	rectum	?	yes	rectum	no
37	68	16	yes	yes		7	yes	DC	no
38	52	multiple	y	yes	right	4	no		no
39	54	30+	yes	yes	left	6	no		no
40	42	multiple	yes	yes	all colon	18	no		yes
41	42	30+			all colon	13	no		no

Table 4.2 Clinical Features of Multiple Adenoma Patients from the United Kingdom in Relation to Germline *MYH* Mutation Status.

Patients	Germline MHY mutation status	Negative (percentage)	Single mutation (percent)	Bi-allelic mutation (percent)
Total Number		140	6	10
Age at presentation	**Known**	139 (99)	6 (100)	10 (100)
	Median	56	64	50
	Range	18-77	25-72	16-59
Polyp number	**Precise count given**	114 (81)	6 (100)	9 (90)
	Median	7	4	40
	Range	3-100	3-12	18-100
Colorectal Cancer	**Yes**	19 (14)	2 (33)	6 (60)
	None Reported	121 (86)	4 (67)	4 (40)
Family history of colorectal cancer	**Positive**	83 (59)	4 (67)	7 (70)
	None reported	57 (41)	2 (33)	3 (30)

Table 4.3 Multiple Adenoma Patients from the United Kingdom with Germline *MYH* Mutations

Note that two of the single mutation carriers had novel MYH variants, R83X (247 C>T) and R295C (883 C>T). It is not known whether or not these variants would be pathogenic as compound heterozygotes or homozygotes, although R83X is likely to be so. CRC = colorectal cancer; N = none reported; n/d = none detected

Patient ID	MYH mutation 1	MYH mutation 2	Gender	Age at diagnosis	Polyp number	CRC	Family history of adenomas and/or CRC	Extra-colonic cancer
IGB	Y165C	Y165C	M	52	40	Y	Y	N
II PB	Y165C	G382D	F	45	100	Y	N	N
III IF	Y165C	nt 1419 delC	F	57	18	Y	Y	N
IV VB	nt 1103 delC	G382D	M	55	70	N	Y	N
V ASM	G382D	G382D	M	56	40	Y	Y	N
VI AMc	G382D	G382D	F	59	100	N	Y	N
VII	Y165C	G382D	M	45	50	N	Y	N
VIII	Y165C	G382D	M	15	39	N	N	Y Gastric
IX	E466X	E466X	M	34	30	Y	Y	N
X	Y165C	G382D	M	48	40	Y	N	N
XI	R83X	n/d	F	69	6	Y	Y	N
XII	Y165C	n/d	M	72	3	N	Y	N
XIII	Y165C	n/d	F	58	5	N	Y	N
XIV	R295C	n/d	F	25	3	N	N	N
XV	G382D	n/d	M	52	3	Y	Y	N
XVI	G382D	n/d	F	70	12	N	N	N

Table 4.4 Clinical Features of Classical Adenomatous Polyposis Patients in Relation to Germline *MYH* Mutation Status

Patients	Germline MHY mutation status	Negative (percentage)	Single mutation (percent)	Bi-allelic mutation (percent)
Total Number		95	4	8
Age at presentation	**Known**	55 (58)	4 (100)	8 (100)
	Median	30	31	47.5
	Range	7-72	30-54	30-70
Polyp number	**100-1000**	68 (72)	30-54	30-70
	>1000	27 (28)	0 (0)	0 (0)
Family history of colorectal cancer	**Positive**	29 (31)	0 (0)	4 (50)
	None reported	66 (69)	4 (100)	4 (50)

Table 4.5 Classical Adenomatous Polyposis Patients with Germline *MYH* mutations

CRC = colorectal cancer; CHRPE = congenital hypertrophy of the retinal pigment epithelium;

N = none reported; n/d = none detected

Patient ID	**MYH mutation 1**	**MYH mutation 2**	**Gender**	**Age at diagnosis**	**Polyp number**	**CRC**	**Family history**	**Extracolonic features**
1	Y165C	Y165C	M	41	100-1000	N	Y	CHRPE
II	Y165C	G382D	F	50	100-1000	N	Y	N
III	G392D	G382D	M	30	100-1000	N	N	duodenal adenomas
IV	nt252 delG	137insIW	M	38	100-1000	Y	N	duodenal adenomas
V	Y165C	Y165C	F	45	100-1000	Y	Y	N
VI	Y165C	V232F	M	70	100-1000	N	N	N
VII	Y165C	G382D	M	51	210	Y	N	N
VIII	Y165C	G382D	F	69	115	Y	Y	N
IX	G382D	n/d	M	32	100-1000	N	Y	N
X	I209V	n/d	F	54	100-1000	N	N	osteoma
XI	Y165C	n/d	M	30	100-1000	N	N	N
XII	Y165C	n/d	M	30	750	N	Y	N

Table 4.6 Somatic APC Mutations in Cancers and Adenomas from Biallelic *MYH* Mutation Carriers.

Patient ID	MYH mutation 1	MYH mutation 2	Tumour type	Mutation
VB	nt 1103 delC	G382D	TA* TAx3 TA	E1461X S1315X E1560X
PB	Y165C	G382D	TA TAx3	E1560X S1315X
IF	nt 1419 delC	Y165C	TAx3 TAx2	E1560X S1315X
JC	G382D	G382D	CA**+TAx3	E1560X
697	G382D	G382D	CA CA	E868X E1552X
761	Y165C	G382D	CA	E1353X
4303	Y165C	Y165C	TA	S1315X
965	Y165C	G382D	CA	E1538X

**CA = Cancer

*TA=Tubular Adenoma

Table 4.7 Oligonucleotide Primers Used for Screening *MYH*

Exon	Primer sequence	Size (bp)	Label	Annealing T	Mg conc.
1	tgaaggctacctctgggaag aggagacggaccgcaagt	141	FAM	55	1
2	ggctgggtctttttgtttca ggccacaacctagttcctt	164	HEX	60	1
3A	ctgtgtcccaagaccctgat ttggtcgtaccagttagca	187	HEX	55	3
3B	agctgaagtcacagccttcc caccactgtccctgctc	110	FAM	60	1
4	cctccaccctaactcctcatc aaagtggccctgctctcag	110	FAM	55	1
5	caggtcagcagtgcctcat gtctgaccatgacccttcc	152	FAM	60	2
6	gtctctttctgcctgcctgt tcacccgtccagtccctctat	125	FAM	60	2
7	cgggtgatctctttgacctc gttcctaccctcctgccatc	134	FAM	60	2
8	tcttgagtcttgcactccaatc	164	HEX	55	1
9	gctaactctttggcccctct cacccttgttaccccaacat	150	FAM	60	3
10	ctgcttcaccagcagtgttcc gaccttctcactgccccttcc	208	HEX	60	2
11	acactcaaccctgtgcctct ggaatggggcttctgactg	147	FAM	60	1
12A	cttggcttgagtagggttcg ggctgttccagaacacaggt	179	HEX	60	1
12B	gagtggtcaacttccccaga cacgcccagtatccaggta	146	FAM	60	1
13	agggaatcggcagctgag gctattccgctgctcactta	209	HEX	60	1
14	aggcctatttgaacccctttg caacaaagacaacaaaggtagtgc	210	HEX	60	1
15	ccctcacctccctgtcttct tgttcacccagacattcgtt	159	HEX	55	3
16A	ctacaaggcctccctccttc gctgcactgttgaggctgt	158	HEX	55	1
16B	gctgcactgttgaggctgt acatagcgagacccccatct	181	HEX	60	2

Table 4.8 Oligonucleotide Primers for Screening *OGGI*

Exon	Primer sequence	Size (bp)	Label	Annealing T	Mg conc
1	gggtaggcggggctactac ccccaaattcctttgtaccc	266	HEX	55	1
2	aattgagtgccagggttgtc ctaaccccagcccaggtc	313	FAM	55	1
3	cagcaggtacctctcctaccc tcttgaaagctgatggaagg	269	FAM	55	2
4	ttgaagatgcctgatgcttg tagagagggcagctcctacc	258	HEX	55	1
5	tcttccacaagggctcattc tctaccatcccagcccact	232	HEX	55	1
6	tcacagaaggggtcagataactt ggctggaagagtcctttaggg	197	HEX	60	3
7	gaccccagtgtaccctcctc atatccccaccccatctt	311	FAM	55	1
8	cattattccgctatgcctca caacaaacactcccaacacct	189	FAM	55	1
9	attctccatgctgccttcct gtaagctggcttgcatcaca	346	FAM	55	1

Table 4.9 Oligonucleotide Primers for Screening *MTH1*

Exon	Primer sequence	Size (bp)	Label	Annealing T	Mg conc
0	gccttatcgcaaaggacagag ccgacctccaggggaaaaat	186	HEX	55	2
1	tgactctgccctctcacctt cggttctatggccagacct	224	FAM	60	1
2	tccctgggctgtgtgtagat agacaggccctgtgagact	270	HEX	60	1
3	ctcttcccccattggtacag ctgttcagcagccacgtct	240	FAM	60	1

Table 4.10 Oligonucleotide Primers for Screening *APC* in Paraffin-Derived Tissue

Exon	Primer sequence	Size (bp)	Label	Annealing T	Mg conc
15 F-G	caagcagtgagaatacgtcca ttcttggttaatagaagaaactttgc	134	FAM	55	1.5
15 G	gaagtggtcagcctcaaaag agaatctgcttcctgtgtcg	178	HEX	55	1.5
15 G	ttcattatcatcttgtcatcagc ggatttggttctagggtgct	177	FAM	55	1.5
15 G-H	gatcctgtgagcgaagttcc ctgagcaccactttggagg	149	HEX	55	1.5
15 G-H	agaatcagccaaggcacaaag gcaatcgaacgactctcaaa	173	FAM	55	1.5
15 H	cactatgttcaggagacccca tggaagatcactggggctta	149	FAM	55	1.5
15 H	gtgaaccatgcagtggaatg acttctcgcttggtttgagc	135	HEX	55	1.5
15 H-I	aaacacctccaccacctcct agcatctggaagaacctgga	145	HEX	55	1.5
15 I	cctaaaaataaagcacctacgctg cactcaggctggatgaacaa	165	FAM	55	3
15 I	cacttttgccacggaaagta ggctgctctgattctgtttc	158	HEX	55	2
15 I	caggaaaatgacaatgggaat tgtattatttctgccatgcca	126	FAM	55	2
15 I-J	ggacctattagatgattcagatgatg ctgtggcaaggaaaccaagt	102	HEX	55	2

Figure 4. 1 Pedigree family I

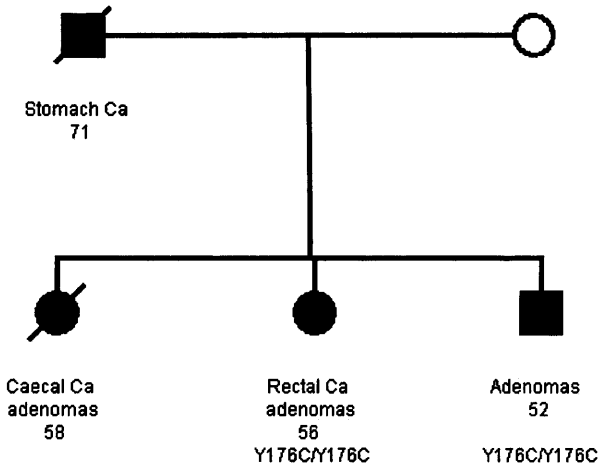


Figure 4. 2 Family II

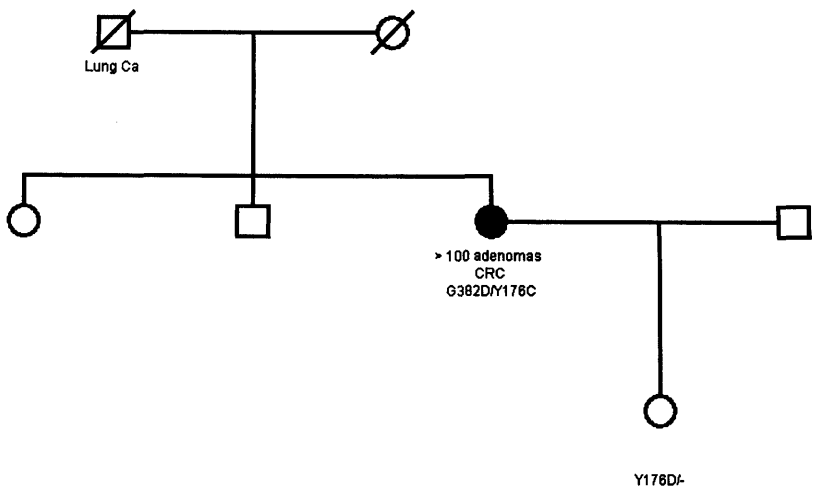


Figure 4. 3 Family III

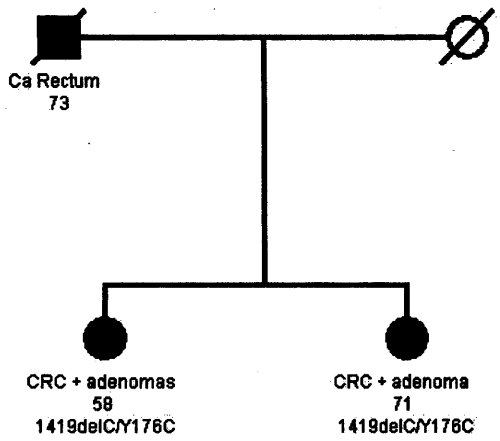
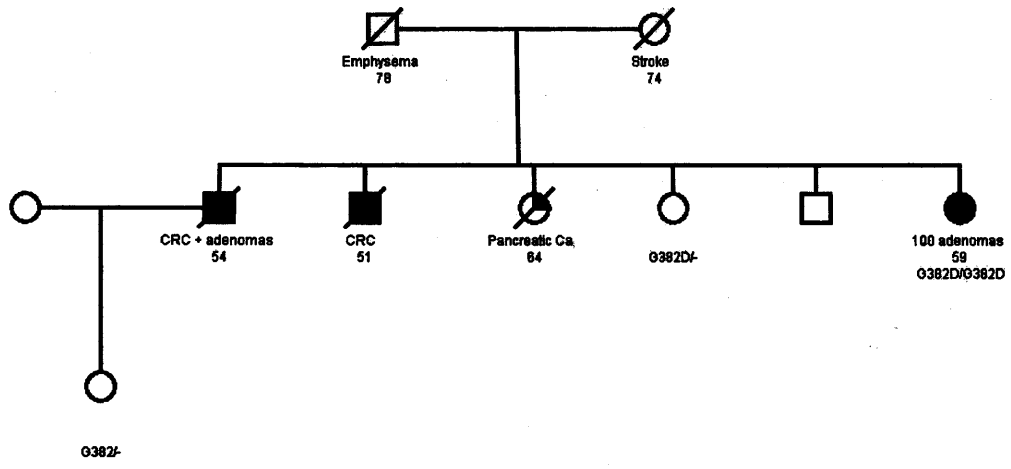


Figure 4. 4 Family IV



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Chapter 5 Pathways of Carcinogenesis in *MYH* associated tumours

5.1 Introduction

Colorectal carcinomas develop according to varying genetic pathways or routes (Chung 2000). The most common pathway is characterised by mutations of the *APC* and *TP53* genes, by 18q allelic loss, by mutation of K-ras and *SMAD4* in some cases, and by an aneuploid/polyploid karyotype (Vogelstein, Fearon et al. 1988). These tumours are often said to have followed the CIN (chromosomal instability) pathway. Other cancers are characterised by microsatellite instability (MSI+), aberrant DNA mismatch repair, a near-diploid karyotype and low levels of *TP53*, *SMAD4* and K-ras mutations, but higher frequencies of *BAX, TGFBIIR* and *BRAF* mutation (Rajagopalan, Bardelli et al. 2002), (Young, Simms et al. 2001). Such cancers include those occurring in HNPCC kindreds and sporadic colorectal cancers with somatic epigenetic silencing of *MLH1*. Still further colorectal cancers have neither MSI nor an aneuploid/polyploid karyotype and are termed MSI-CIN- (Georgiades, Curtis et al. 1999). These cancers appear to form a minority of around 10%. Superimposed on these three pathways are further levels of complexity; some colorectal cancers, for example, may have tendency to high levels of promoter methylation (so-called CIMP+ pathway) (Toyota, Ahuja et al. 1999).

Germline *APC* mutations generally lead to a phenotype of profuse colonic polyposis (FAP). One ore more of these adenomatous polyps usually progresses to cancer by the fourth or fifth decade , probably owing to second hits at the remaining *APC* allele and mutations in the same genes involved in the development of sporadic CIN+ colorectal cancers (Debinski, Love et al. 1996; Tomlinson, Ilyas et al. 1998; Shibata and Aaltonen 2001). It is likely – although not conclusively demonstrated – that FAP polyps can become cancerous owing to progression along any of the CIN+, MSI- CIN- pathways (Konishi, Kikuchi-Yanoshita et al. 1996). In HNPCC, by contrast to FAP, the phenotype is predominantly one of colorectal carcinoma, with only a small increase in incidence of colorectal adenomas (Lynch 1999).

As described in chapter three, MYH associated polyposis (MAP) is a newly described syndrome predisposing to colorectal adenomas (multiple or polyposis). Phenotype is often indistinguishable from a classical or attenuated form of FAP, but MAP appears to be inherited as a recessive trait, with consequent implications for the risk of disease in other

family members (Sieber, Lipton et al. 2003). *MYH* encodes a glycosylase which is involved in base excision repair and primarily targets oxidative DNA damage to guanine residues. In keeping with the role of MYH, colorectal tumours from MAP patients show an excess of G→T transversion mutations in the *APC* gene owing to the failure to repair lesions induced by the variant base 8-oxo-guanine (Al-Tassan, Chmiel et al. 2002; Sieber, Lipton et al. 2003). Whilst changes in MYH expression may have some role in the pathogenesis of sporadic colorectal tumours, there is currently no evidence to show that the gene is mutated or silenced in bowel cancers outside MAP (Halford, Rowan et al. 2003).

MYH is the first base excision repair gene to be shown to predispose to colorectal cancer when mutated in the germline. It is, in many ways, an unexpected gene to cause colorectal polyposis. Many puzzles regarding its role in tumourigenesis remain. It is not clear, for example, why germline *MYH* mutations lead to tumours of the gastrointestinal tract, or why MAP differs in its phenotype and inheritance from HNPCC. In order to gain further clues to explain how germline *MYH* mutations lead to a phenotype of multiple colorectal tumours and cancer, a study was made of the somatic genetic changes in 130 colorectal adenomas and 19 carcinomas from 22 MAP patients.

5.2 Methods

5.2.1 Selection of MAP Tumours

Collection of tumours, DNA extraction, sequencing and immunohistochemistry were done by me. Miss Victoria Johnson helped in performing immunohistochemistry for p53 and beta-catenin.

The 22 patients with bi-allelic germline *MYH* were identified through Family Cancer clinics and Polyposis Units in the United Kingdom (St Mark's Hospital, Harrow), Finland, Denmark and Switzerland. *MYH* mutation testing had been done by myself and Dr Oliver Sieber as part of a previous study (18 patients) and a population based study performed in Finland (four patients) (Enholm, Hienonen et al. 2003). Mutation testing for the latter was performed by me. All patients were known to have multiple colorectal adenomas, although precise adenoma counts were not available for some patients. Most of our patients had developed

one or more colorectal cancers, although only 19/26 of these tumours were available for study (Table 5.1).

5.2.2 DNA Extraction

Constitutional DNA was extracted from peripheral blood lymphocytes by standard methods. Both fresh-frozen and fixed, paraffin-embedded tumour tissues were used for the analysis, depending on their availability. DNA was extracted using standard methods (chapter 2.).

5.2.3 Mutation Analysis

For mutation analysis of *APC*, *SMAD4*, *beta-catenin* and *TP53* in tumour DNA, F-SSCP analysis was used. dHPLC was additionally used for *APC*. Primer pairs for *APC* codons 1219 to 1609 (exon 15 regions F to I) were used as in chapter four. Where DNA derived from fresh-frozen tissue was available, the tumour was analysed for *APC* mutations from exon 15A to 15L. Primer pairs were similarly designed for the coding regions and exon-intron boundaries of *TP53* exons 4 to 8, *SMAD4* (all exons and flanking regions), *beta-catenin* exon 3, K-ras exon 3 and *BRAF* exon 14 (Tables 5.2-6).

For F-SSCP analysis, each 25 µlPCR reaction contained 1xPCR reaction buffer without $MgCl_2$, 200 µM dNTPs, 200 nM of each primers, 50 ng of genomic DNA and 1U of *Taq* DNA polymerase (Qiagen), and the PCR conditions consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C or 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min. The resulting PCR products were screened for variants by being run at 18°C and 24°C on the ABI 3100 and analysed using Genotyper 2.5 software (Perkin-Elmer Applied Biosystems). The latter was performed in the CRUK Equipment Park.

DHPLC was performed using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK). The same primer set was used as for the F-SSCP analysis. To enhance the formation of the hetroduplexes prior to analysis, the PCR products were denatured at 94°C and re-annealed by cooling to 50°C at a rate of 1°C per minute. dHPCL was carried out at the melting temperature predicted by Wavemaker (version 4.0) software (Transgenomic) with a 12% acetonitrile gradient over 2.5 minutes. DHPLC analysis was carried out by the CRUK Equipment Park.

For tumour samples showing aberrant SSCP or dHPLC bands, the relevant region of the gene was re-sequenced directly from a new PCR product alongside constitutional DNA. For samples without mutations seen on standard sequencing, TA cloning was performed on the PCR fragment using pGEM-T Easy Vector System (Promega) following the manufacturer's instructions. A minimum of 10 clones was then sequenced.

K-ras exon one was sequenced directly in 15 cancers and a subset of 30 adenomas. *BRAF* exon 14 (the region around the mutation hotspot at codon 599, GTG (Val) was sequenced directly in a subset of 12 cancers and 55 adenomas.

5.2.4 LOH and MSI Analysis

For loss of heterozygosity (LOH) and MSI analyses, the tumour DNA was PCR-amplified alongside the constitutional DNA from the patient using microsatellite markers MYCL, D2S123, D5S346, D10S197, D15S659, D18S69, D18S487 and D20S100, with the forward primer fluorescently labeled with FAM, TET or HEX. For LOH, areas under the peaks (including stutter bands) were compared for all informative (heterozygous) markers. LOH was reported where normal:tumour peak areas were less than 0.5, or greater than two, thereby allowing for contaminating normal tissue within the microdissected tumour.

For assessing MSI, the eight markers above were used, together with mononucleotide repeats TGFBIIR (transforming growth factor-beta type II receptor) and BAT26. A tumour was considered MSI+ if four or more markers showed novel alleles. Whilst the significance of lower levels of MSI is unclear, we noted tumours which had one to three markers unstable.

5.2.4 Immunohistochemistry

Immunohistochemistry was performed for TP53 over-expression and aberrant (nuclear) beta-catenin expression. Five μm tumour sections were analysed using the M7001 (Dako) and GC19220 (Transduction Laboratories) antibodies respectively at 1/100 dilution (beta-catenin) or 1/1000 dilution (*TP53*) after microwaving the sections for 10 minutes. After counterstaining with haematoxylin, the slides were examined by three independent observers (I, V Johnson and A.T. Eftekhar Sadat). Beta-catenin expression was scored as positive if

nuclear staining was present in more than 10% of dysplastic cells. For TP53, a tumour was scored as positive if there was no staining of normal tissue and more than 20% of dysplastic nuclei were stained. Tumours were reported as negative for p53 if normal tissue showed no staining and <20% of dysplastic nuclei were stained. Tumours with other p53 staining patterns were excluded from analysis.

5.2.5 Flow Cytometry

Flow cytometry, was performed on tumour/normal tissue pairs with the help of the FACS laboratory and CRUK, Lincoln's Inn Fields (see chapter 2).

5.3 Results

5.3.1 Clinical Features

The 22 patients (Table 5.1) presented at a mean age of 55 years (median 55, range 38-71). The mean number of adenomas in the 13 patients for whom precise counts were obtained was 70 (median 70, range 5-210). Adenomas were of tubular or tubulovillous morphology, with occasional hyperplastic polyps and serrated adenomas were also present. Microadenomas or oligocryptal adenomas were present in some patients. Several patients were seen to have areas of flat dysplastic mucosa without histological features of discrete adenomas. For all patients there was great variation in the size of adenomas (range one to 40mm where reported). No patient in this series was known to have developed extra-colonic tumours. Fourteen patients had developed one colorectal cancer and six had developed two cancers (five synchronous, one metachronous). Two patients had developed adenomas only. The colorectal cancers in the study were predominantly left-sided (17/24 reported), with most occurring in the rectum (Table 5.1). In five of the patients with two cancers, both occurred in the recto-sigmoid region. The cancers had no characteristic histological pattern, with only three reported as showing a mucinous histology. Where it was reported, grade 2 cancers predominated, with 2/23 (9%) grade 1, 16 (70%) grade 2 and 5 (22%) grade 3 histology. Stage varied widely amongst cancers (Table 1). Carcinomas were present together with adenomatous elements in many specimens suggesting transformation of adenoma into carcinoma.

5.3.2 Molecular Features and Somatic Mutation Profile of Tumours

All protein-truncating *APC* mutations in our MAP tumours were G>T transversion mutations. Only one tumour showed LOH at *APC*. Although the quantity of tumour tissue available permitted only part of the *APC* gene to be screened, 22 of 105 (21%) adenomas and six of 14 (43%) cancers had at least one *APC* mutation (Table 5.2). The most commonly found changes were the nonsense mutations S1315X (nine adenomas) and E1560X (one cancer and 11 adenomas). It is noteworthy in passing that four of the 19 MAP patients harboured the germline missense *APC* variant E1317Q (Lamlum, Al Tassan et al. 2000). This variant, which has a population frequency of about 1% is of uncertain significance, but also provided the original cause for investigation of the first MAP family reported (Al-Tassan, 2002). None of the 105 adenomas and 14 colorectal cancers that underwent F-SSCP analysis of *beta-catenin* exon 3 showed any mutations. Immunohistochemistry revealed aberrant (nuclear) beta-catenin staining in 12/17 (71%) cancers and 16/38 (42%) adenomas studied, consistent with loss of APC function in these lesions (Table 5.7).

For K-ras, the same mutation (GGT→TGT, G12C) was found in 10 of 15 (67%) cancers and 13 of 30 (43%) adenomas tested (Figure 5.1, Table 2). No other K-ras mutations were found.

No *BRAF* mutations were found in exon 14, the site of the most common variant V600E.

We tested 14 cancers and 115 adenomas for mutations in *TP53* exons four to eight. Three changes were found, all in carcinomas. One mutation, delAGTACTGT nt380, is highly likely to be pathogenic. Two changes, P152T (C→A, nt463) and F134V (T→G), have previously been reported as rare changes in cancers, and have potential functional importance (Beroud and Soussi 2003). Of these three mutations, only P152T involves a G>T change. Immunohistochemistry revealed abnormal nuclear p53 staining in 8/15 (53%) cancers and in 2/41 (5%) adenomas studied. *TP53* mutations and immunohistochemistry were concordant (Table 5.2).

Four out of 12 informative cancers (33%) and 14/79 (18%) informative adenomas showed LOH at one or more 18q markers. We found no coding *SMAD4* changes. No mutations were found in the *TGFBIIR* repetitive oligonucleotide tract.

None of the 149 MAP tumours showed MSI (Table 5.2). In keeping with this, none of 12 adenomas and nine cancers studied showed loss of MLH1, MSH2 or MSH6 expression (details not shown). Nine adenomas (7%) and one cancer (6%) showed microsatellite slippage at a single marker, and one other cancer showed slippage at two markers. We deemed that these data did not suggest any particular tendency to MSI-low in our tumour set (Halford, Sasieni et al. 2002; Laiho, Launonen et al. 2002). LOH was also uncommon in the cancers at the markers studied (maximum of 4% of informative cases with loss at each marker, excluding the chromosome 18 microsatellites) (Table 5.7).

Consistent with the relatively low frequency of LOH, flow cytometry showed that 12 out of 13 (92%) MAP cancers tested were near-diploid (Figure 5.2). The remaining cancer appeared near-tetraploid. This cancer had the characteristic K-ras mutation and 18q LOH, but no other detectable genetic changes (Table 5.2). In order to provide corroboration of the flow cytometry, two of the near-diploid cancers were screened for changes using a genomic microarray, and no detectable changes were found (data not shown).

As expected, cancers showed a higher frequency of changes overall than adenomas. For certain changes, such as *APC* mutations, this may, at least in part, have reflected the problems of studying smaller lesions using molecular methods. Methodological problems could not, however, readily explain other differences between adenomas and carcinomas, such as the more frequent aberrant expression of beta-catenin and p53 in the malignant lesions. We found no other associations between clinical, pathological and molecular data, including the presence of K-ras mutation.

We compared the features of our MAP tumours with those of 107 unselected sporadic colorectal cancers studied in our laboratory using similar methods (Table 5.8). Whilst statistical comparisons must be undertaken cautiously – partly because sporadic cancers comprise a mixture of MSI+ CIN-, MSI- CIN+ and MSI- CIN- lesions – our unselected series had molecular features in accordance with findings from other studies (details below) and had been analysed using the same methods as the MAP cancers (Miyaki, Konishi et al. 1994; Konishi, Kikuchi-Yanoshita et al. 1996; Fujiwara, Stolker et al. 1998; Sakao, Noro et al. 1998). Certain features of the MAP tumours were striking. Whilst no specific histological features distinguished the MAP tumours (details not shown), MAP carcinomas occurred at a relatively young age (median 55, range 38-71) compared to the sporadic group

(median 66, mean 66, range 27-103) ((χ^2_1=15.9, p=0.0001, Mann-Whitney test). *APC* mutations occurred at a similar frequency in MAP and sporadic cancers (6/14 versus 44/99, p=0.58, Fisher's exact test). As expected, the mutation spectrum in the *APC* gene was overwhelmingly biased to G→T in MAP cancers compared with the sporadic lesions; G→T changes comprised 6/6 truncating mutations in the MAP cancers (and 22/22 in MAP adenomas) compared with 4/44 in the sporadic cancers (p=0.0001, Fisher's exact test). *Beta-catenin* mutations were very uncommon in both the MAP tumours (0%) and the sporadic cancers (1%), consistent with other studies (Sparks, Morin et al. 1998; Hao, Frayling et al. 2002). Nuclear beta-catenin was seen in 47/72 sporadic and 12/17 MAP cancers (p=0.46, Fisher's exact test).

K-ras mutations were present in 9/14 MAP cancers (and 13/30 MAP adenomas) and 33/104 sporadic cancers (p=0.02, Fisher's exact test), but the spectrum in the former was restricted to a single change in codon 12 which was found in only 3/33 sporadic colorectal cancers (p=0.0001, Fisher's exact test). This comparison holds true when mutation frequencies from the RASCAL study are compared with those of the MAP cancers (Andreyev, Norman et al. 2001). Three of 14 MAP cancers had pathogenic *TP53* mutations compared with 33/92 sporadic cancers (p=0.23 Fisher's exact test). Abnormal p53 over-expression was similar (p=0.50, Fisher's exact test) in MAP cancers (8/15) and sporadic lesions 26/53). *SMAD4* mutations were not found in the MAP cancers, although such mutations were also uncommon in the sporadic cancers (p=0.89, Fisher's exact test). 18q LOH was as common in the MAP as the sporadic cancers (7/15 versus 33/74, p=0.55, Fisher's exact test). MSI was notably absent from the MAP cancers, although this was not significantly different from the 11% (12/107 of MSI cancers in our set of unselected colorectal carcinomas (p+0.127, Fisher's exact test). Diploidy was much more common in the MAP, occurring in 12/13 cancers compared with 43/90 sporadic lesions (p=0.002, Fisher's exact test).

5.4 Discussion

This study suggests that colorectal tumours developing in patients with bi-allelic germline *MYH* mutations follow a distinct genetic pathway. Although MAP is an uncommon condition and hence, at the present time there are not a large number of DNA samples available from MAP cancers and adenomas, this data does allow some firm conclusions to be

drawn. The spectrum of somatic mutations in the *APC* gene largely comprises G→T transversion mutations. These mutations are present in both adenomas and carcinomas. They are particularly frequent at codons S1315X and at the known hotspot, E1560X, although we have not found mutations at the previously reported hotspots of codons 836 and 932 (Al-Tassan, Chmiel et al. 2002). The later two mutations were found in a single family however and may not be representative of the MAP cancer mutation spectrum as a whole. The high frequency of the germline *APC* variant E1317Q (21% compared to the population frequency of 1%) is an interesting if unexplained finding. Lamlum et al found this variant at a frequency of 8% in a cohort of 164 multiple adenoma patients and concluded that is was associated with the multiple adenoma phenotype (Lamlum, Al Tassan et al. 2000). It may be that the raised frequency in that population was an indication that there were a significant proportion of MAP patients within the cohort. It is possible that the association of E1317Q with colorectal tumours may be due to its co-occurrence with *MYH* mutations.

From the data is appears that the genetic instability resulting from MYH deficiency not only targets *APC*, but also affects other genes. K-ras mutations were common in tumours from these MAP patients and were all the same G12C (G→T) change. Previous reports had suggested that in the MYH deficient genome, AGAA or TGAA motifs were preferentially targeted. Such motifs occur with great frequency in *APC*. This motif is not present at the critical sites within K-ras, but the G→T mutations in K-ras preferentially involved the first guanine reside of codon 12 (TGGT), despite there being three other guanines within codons 12 and 13 which could have served as targets for mutation. Thus, whilst *MYH* mutations lead to an excess of G→T changes, the surrounding sequence affects the probability of this change, and the resulting somatic mutation spectrum reflects both selection and hypermutation. It is interesting to note that in a large study of 1993 incident colorectal cancer cases and 22410 controls, a family history of colorectal cancer was not associated with k-ras mutations overall but was associated with G→T mutations in codon 12 (Slattery, Curtin et al. 2002).

The role of *TP53* in the MAP patients' tumours is unclear. TP53 over-expression was about as frequent as in sporadic cancers. *TP53* mutations were detected in MAP cancers, but – unlike *APC* and K-ras – there was no clear bias to G→T changes in *TP53*. *SMAD4* mutations and TGFBIIR mutations tract appear to play at most a minor role in the genesis of MAP tumours, although 18q LOH was as common as in sporadic cancers. It is not known as to

whether or not other components of the transforming growth factor beta pathway are inactivated in MAP cancers. This sets these BER tumours clearly apart from HNPCC tumours due to MMR which frequently show *TGFBIIR* variants and rarely show *TP53* changes (Konishi, Kikuchi-Yanoshita et al. 1996) .

All of our MAP patients' cancers were MSI- and almost all were near-diploid (CIN-). It may be the case that MYH deficiency confers sufficient genomic instability to make additional forms of instability unnecessary or disadvantageous for tumourigenesis. We found no evidence of unusual levels of MSI-low in MAP cancers, even though it has been suggested that MYH deficiency might lead to MSI-low because the MutSα complex is involved in mismatch recognition for both base excision repair and mismatch repair, and hence might be overloaded by an excess of G→T changes (Gu, Parker et al. 2002).

It can be concluded that the MAP pathway of carcinogenesis is distinct from both the CIN and MSI pathways (Table 5.9). MAP tumours have high frequencies of *APC* mutation and low frequencies of beta-catenin mutations. Like MSI+ cancers, MAP cancers tend to be near-diploid and to have low frequencies of overall LOH. MAP cancers do not, however, have TGFBIIR mutations, as sporadic MSI+ cancers do, probably as an alternative to *SMAD4* mutations. We have not determined whether or not MAP cancers harbour *BAX* mutations, as MSI+ cancers do, largely owing to the presence of mononucleotide tracts within the gene (Simms, Radford-Smith et al. 1998). We do not predict that these genes would be particularly susceptible to the G→T changes found in MAP patients. Many of these are, in fact, polyA tracts. The relatively high frequencies of k-ras mutations and 18q LOH in MAP cancers more closely resemble tumours following the CIN pathway than the MSI pathway. With respect to K-ras, controversy does exist regarding mutation frequency in HNPCC cancers and some investigators put this as high as 40% (Oliveira, Westra et al. 2004).

Overall, we can hypothesise that despite overlap in the genes which are mutated, colorectal cancers generally have only one major type of instability, whether CIN, MSI or, in MAP, base excision repair deficiency. It is not yet known whether aneuploidy and polyploidy are rare in MSI+ and MAP cancers because there is no 'need' for LOH as frameshift and G→T changes respectively occur at sufficiently high frequencies – or because too much genetic instability harms the cell or prevents survival and proliferation. For MSI+ cancers, both sporadic and HNPCC, the subsequent genetic pathways can partly be explained because

certain genes with short repetitive sequences are susceptible to mutation. There is currently no good evidence to show that certain genes are especially prone to the incorporation of 8-oxo-guanine or to resistance to repair of an 8-oxoG:A mismatch, although certain sequences are preferentially mutated in MAP tumours. The possibility remains that MAP cancers resemble MSI+ cancers in their gross genetic features such as karyotype, but are more like CIN+ cancers in the genes which are mutated.

With regard to the clinical syndrome of MAP, this study highlights several important points. Distinct from HNPCC, and sporadic MSH+ cancers, tumours in MAP tend to be left sided and rarely mucinous. The number of persons diagnosed with synchronous cancers is likely to relate to multiple pre-neoplastic lesions and late diagnosis. A similar scenario may be seen in FAP. The risk of metachronous colorectal cancer in HNPCC remains high after an initial CRC diagnosis in HNPCC with new adenomas rapidly forming and malignancy developing. It is currently not known what the risk of new advanced adenomas is in MAP patients after initial clearance of polyps and cancers. Thus in phenotype as in genotype this syndrome seems to fall between HNPCC and FAP with the management paradigm of neither seeming entirely appropriate.

Table 5.1 Patient and Tumour Characteristics

CRC = colorectal cancer, R=rectum, S=sigmoid colon, H=hepatic flexure, C=caecum, SF=splenic flexure, TA=tubular adenoma, TVA=tubulovillous adenomas, VA=villous adenomas, HPP=hyperplastic polyps, N/R=not reported

Patient	MYH mutation 1	MYH mutation 2	Age at dx	No. of CRC'S	CRC stage	CRC grade	CRC site	No. of adenomas	Size of adenomas	Predominant adenoma pathology
1	DelC, nt 1452	Y165C	57	2	B and A	2 (both)	R (both)	28	2-30mm	TA and TVA, microadenomas
2	DelC, nt 1452	Y165C	71	2	B (both)	2 (both)	R and S	18	N/R	TVA and HPP
3	G382D	G382D	49	0				100	2-5mm	TA
4	G382D	G382D	51	1	C	2	H	'multiple'	3-25mm	TA
5	DelC, nt 1136	G382D	55	0				70	1-25mm	TA and TVA, microadenomas
6	G382D	G382D	56	1	C	1 (mucinous)	C	40	2-30mm	TA
7	Y165C	Y165C	52	1	N/R	N/R	N/R	40	1-5mm	TVA
8	Y165C	Y165C	55	2	B and A	2 (mucinous) and 2	R	'multiple'	5-30mm	VA and TVA
9	Y165C	G382D	69	1	B	2 (mucinous)	C	115	1-40mm	TA, TVA and HPP
10	Y165C	Y165C	58	2	C (both)	2 and 3	SF and C	'numerous'	2-15mm	TA and TVA
11	Y165C	Y165C	45	1	B	2	R	'numerous'	2-15mm	TA and TVA
12	DelGGA, nt 1395	G382D	53	1	D	3	H	14	2-25mm	TA
13	G382D	G382D	66	1	D	2	R	5	N/R	N/R
14	Y165C	G382D	40	1	D	3	R	'several'	N/R	N/R
15	Y165C	G382D	55	1	D	2	C	80	N/R	N/R

Patient	MYH mutation 1	MYH mutation 2	Age at dx	No. of CRC'S	CRC stage	CRC grade	CRC site	No. of adenomas	Size of adenomas	Predominant adenoma pathology
16	Y165C	G382D	52	1	A	2	R	50-100	N/R	N/R
17	Y165C	G382D	51	2	B and A	1 and 2	S and R	210	5-25mm	TA and TVA
18	Duplication ATGGAT, nt 411	nt 252 delG	38	1	C	N/R	R	>100	N/R	TA and TVA
19	G382D	G382D	58	2	C (both)	3 and 2	R and S	'multiple'	5-30mm	TA and TVA
20	Y165C	G382D	49	1	B	2	C	N/R	N/R	N/R
21	Y165C	G382D	68	1	C	3	S	N/R	N/R	N/R
22	Y165C	G382D	65	1	N/R	N/R	N/R	N/R	N/R	N/R

Table 5.1 (Continued) Patient and tumour characteristics

CRC = colorectal cancer, R=rectum, S=sigmoid colon, H=hepatic flexure, C=caecum, SF=splenic flexure, TA=tubular adenoma, TVA=tubulovillous adenomas, VA=villous adenomas, HPP=hyperplastic polyps, N/R=not reported

Table 5.2 *TP53* Primer Sequences

EXON	SEQUENCE
5A	atctgttcacttgtgccctg ctcacaacctccgtcatgtg
5B	ttgccaactggccaagacct atcagtgaggaatcagaggc
6	gcctctgattcctcactgat ggagggccactgacaacca
7A	cttgccacaggtctccccaa tgatgatggtgaggatgggcc
7B	atctcctaggttggctctgac aggggtcagcggcaagcaga
8	ttccttactgcctcttgctt tgaatctgaggcataactgc

Table 5.3 SMAD4 Primer Sequences

EXON	SEQUENCE
1A	ttgcttcagaaattggaca aaactttcaattgctcttttgc
1B	gcaaaaagagcaattgaaagtt tctgccaccatagagggtat
2A	atataaaagtgtcttgcataatgtgac tggattcacacagacactatcac
2B	tggcctgatcttcacaaaaa tgagatccttttccctttatgttt
3	tgtttcatttgttttccccttt ctgccgctcacacaaactaa
4	gaatatgtgtgcatgactttgagg ttctagaactcacttgtggaagc
5	catgttaatgtcttcttgttcctc ggctgcctacttttctcaa
6	acccatgtgggccttaattt gcccttacaacaaaaacaagag
7	tttactgaaagttttagcattagacaa gcctgtgtttgtcgtttcaa
8	tttctcatgggaggatgttc tgtggacattggagagttga
9A	gcatgctatacaatctgaagtaaaat cgcccagcttctctgtctaa
9B	gggtcaggtgccttagtgac tccttccacccagatttcaa
10A	ggcattggtttttaatgtatgga ctgggccagggatgtttc
10B	caggcggctactgcacaa tgctcaaagaaactaatcaactgag
11A	atcaccctgtccctctgatg ttcaatccagcaaggtgtttc
11B	cggattacccaagacagagc tttgtagtccaccatcctga

Table 5.4 *Beta-catenin* Primer Sequence

EXON	SEQUENCE
3	gaaccagcagaaaagcggctg actcatacaggacrrgggagg

Table 5.5 *BRAF* Primer Sequence

EXON	SEQUENCE
14	tcataatgcttgctctgatagga ggccaaaaatttaatcagtgga

Table 5.6 K-ras Primer Sequence

EXON	SEQUENCE
1	aaggcctgctgaaaatgact agaatggtcctgcaccagtaa

Table 5.8 Features of Sporadic and MAP Cancers

	MAP cancers (%)	**Sporadic cancers (%)**	**P Value**
Median age at diagnosis	55	66	0.0001
MSI+	0/15 (0)	12/107 (11)	0.172
Diploid	9/10 (90)	43/90 (48)	0.011
APC mutations	6/14 (43)	44/99 (44)	0.58
Kras mutations	9/14 (64)	33/104 (32)	0.02
Kras G>T transversions	9/9 (100)	4/44 (9)	0.0001
P53 mutations	3/14 (21)	33/92 (36)	0.39
P53 immunstain	7/12 (58)	26/53 (49)	0.50
B-catenin immunostain	12/14 (86)	47/72 (65)	0.46
18q LOH	4/10 (40)	33/74 (44)	0.53

Table 5.9 Comparison of Genetic Features in the Known Colorectal Cancer 'Pathways'

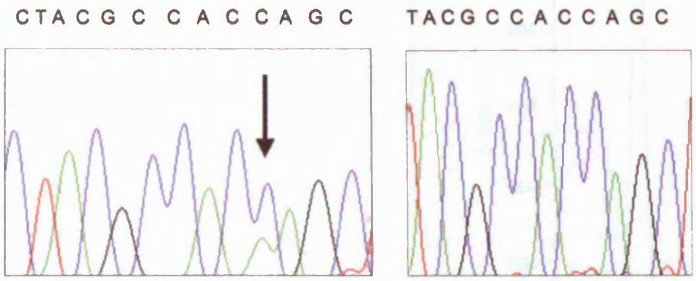
	CIN+	MSI+	BER+
MSI	-	++	-
Ploidy	aneuploid	diploid	diploid
LOH	++	-	-
APC mutation	++	+	++
Kras mutation	++	+	++
P53 mutation	++	+	+
TGFRB mutations	-	++	-

Table 5.7 Molecular data for MAP colorectal carcinomas Blank cells=no result or experiment not done.

Patient	CRC No.	APC mutation	APC LOH	Bcat mutation	Bcat immunohx	K-ras mutation	p53 mutation	p53 immunohx	SMAD4 mutation	18Q LOH	Ploidy
2	CA1	None found	No	None found	+	G12C, G>T	None found		None found	Yes	Polyploid
	CA2	None found	No	None found	+	G12C, G>T	None found		None found	No	Diploid
4	CA	E1560X	No	None found	-	G12C, G>T	F134V, T>G	+	None found	Yes	Diploid
6	CA	None found	No	None found	+	None found	None found	-	None found	No	Diploid
7	CA	None found	No	None found	+	None found	None found	-	None found	No	Diploid
8	CA	None found	No	None found		G12C, G>T	None found		None found	No	
9	CA	E1494X, S1315X	No	None found	-	G12C, G>T	None found	+	None found	No	Diploid
10	CA1	S1315X	No	None found	+	G12C, G>T	None found	+	None found	Yes	Diploid
	CA2	None found	No	None found	+		None found	-	None found	Yes	Diploid
11	CA			None found	+	None found		+			Diploid
12	CA	None found	No	None found	+	G12C, G>T	None found	-	None found	No	Diploid
13	CA	E1552X, E868X		None found	+	None found	None found	+	None found	No	Diploid
14	CA	E1353X		None found		G12C, G>T	delAGTACTGT nt380		None found		Diploid
15	CA	E1353X		None found	+	G12C, G>T	P152T, C>A	+	None found		Diploid
16	CA	None Found		None found	+	None found	None found	+	None found	No	
19	CA				+			_			
20	CA		No		_	G12C, G>T		_		Yes	
21	CA		No		_			+		Yes	
22	CA		No		_			_		Yes	

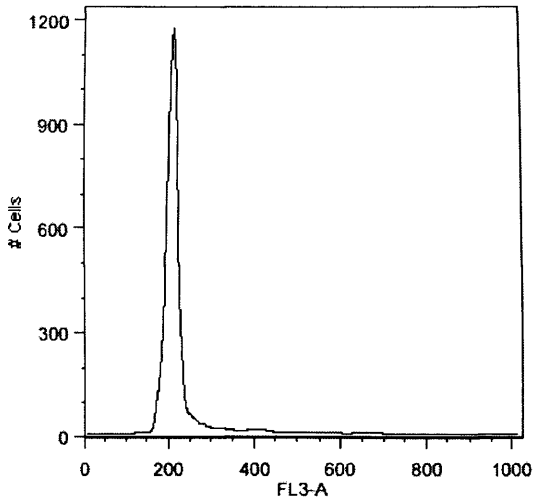
Figure 5. 1Common K-ras Mutation in MAP Tumours

Fig. 2

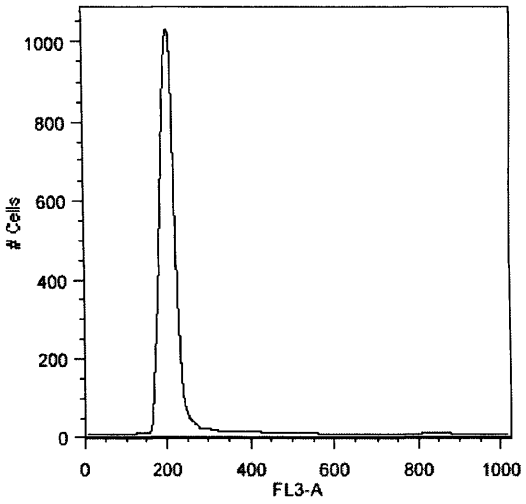


Lipton, L. et al. Cancer Res 2003;63:7595-7599

Figure 5. 2 Flow Cytometry of Cancers from patients 9 and 12 (diploidy)

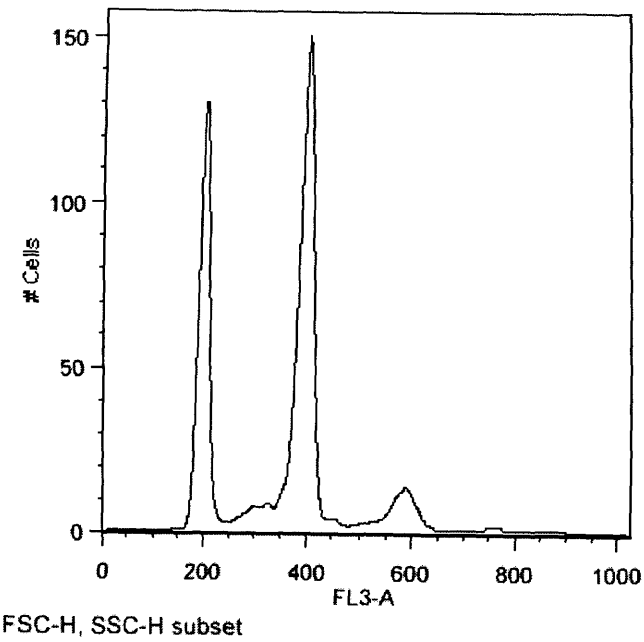


FSC-H, SSC-H subset
1
Event Count: 10645



FSC-H, SSC-H subset
2
Event Count: 11475

Figure 5. 3 Flow Cytometry from Patient 2 (polyploidy)



FSC-H, SSC-H subset
20
Event Count: 2510

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Chapter 6 Molecular Study of Adenomas from Patients with Multiple Adenomas or Polyposis and without Identified Genetic Predisposition

6.1 Introduction

Following the theme of previous work done on MYH associated polyposis (MAP) it became apparent that this syndrome accounted for a significant percentage (30%) of patients with over 15 colorectal adenomas and who did not have detectable mutations in *APC*. It remains apparent that many individuals and families with a multiple adenoma or polyposis phenotype still remain without genetic diagnosis. This makes it almost impossible to give evidence-based advice regarding screening and risk to family members. Because of the striking phenotype of multiple colorectal adenomas I and others felt that other germline changes must exist which could predispose to such disease. A number of strategies may be used to find new genes responsible, yet none is by any means perfect. One method, which has proved fruitful in the past, is the documentation of the 'footsteps' or outcome of the germline genetic disorder in the somatic mutation spectrum of tumours. The mismatch repair (MMR) genes were targeted in HNPCC when it became apparent that instability of microsatellites or repetitive DNA sequences was a hallmark of tumours from families fulfilling the Amsterdam Criteria. Such instability occurs due to defective MMR. In a serendipitous occurrence, the over-representation of G→T transversion mutations in the tumours from a multiple adenoma sibship lead to the investigation of the genes involved in the base excision repair (BER) pathway which usually prevents such mutations. Thus germline missense *MYH* mutations were uncovered.

The *APC* gene frequently undergoes mutation or loss as an initiating event in adenoma formation. It appears to act as a gatekeeper, its loss permitting a cascade of deleterious changes. Thus it forms an excellent starting point to assess the somatic mutation spectra in adenomas from patients who do not have detectable *APC* or *MYH* germline variants yet develop tens to hundreds of adenomas. In FAP tumours *APC* may be lost (LOH) or develop protein-truncating changes, with pathogenic missense mutations being very rare (Ilyas and Tomlinson 1996; Sieber, Heinimann et al. 2002; Crabtree, Sieber et al. 2003). Protein truncating variants tend to occur in the somatic mutation cluster region (MCR), which lies between codons 1286 and 1513 (Rowan, Lamlum et al. 2000). This part of the gene contains

a set of 20-amino-acid repeats thought to be critical for beta-catenin binding and degradation. Such a tight clustering of mutations implies that of all the functions downstream to the MCR which are lost, the loss of functions which are immediately adjacent to the 3' limit of the MCR may be more important in tumour development. A series of three SAMP (Ser-Ala-Met-Pro) repeats which mediate Axin1 binding occur 3' to the MCR and mutations in this area may well be under-recognised. In FAP, the site of 'second hits' is dependent on the site of the germline change (Albuquerque, Breukel et al. 2002). LOH tends to occur in adenomas of persons with germline mutations near codon 1300 and truncating changes in the MCR in those with other germline variants. It has been postulated that a combination of mutational/LOH events that leave one or two intact 20-amino –acid repeat sequences may be optimal for adenoma development (Lamlum, Ilyas et al. 1999; Albuquerque, Breukel et al. 2002; Groves, Lamlum et al. 2002; Crabtree, Sieber et al. 2003). Evidence also exists for a strong association in sporadic colorectal cancers between truncating mutations in codons 1200 to 1400 and loss of the remaining non-mutant allele (Rowan, Lamlum et al. 2000).

As a result of work by myself and others in my laboratory and the St Mark's familial cancer clinic with multiple adenoma patients it has become apparent that a large number of those with higher polyp counts (>15) appear to be isolated cases within the family or have an apparently autosomal recessive family history. Families without known germline mutations in our cohort fall into two broad groups, those with fewer polyps and an autosomal dominant picture of inheritance including colorectal cancer and those with isolated affected persons or sibships with large numbers of polyps. A number of persons when followed for some years develop both hyperplastic and adenomatous polyps. In the current study I have elected to examine the adenomas from those with over five synchronous or metachronous polyps both from isolated and from familial cases.

The major question to be investigated involved the nature of the pathway of tumourigenesis in polyps from multiple adenoma patients without germline *APC/MYH* mutations. Adenomas are known to develop secondary to Wnt pathway disruption due to inactivating *APC* or, more rarely, activating beta-catenin mutations, or to lack of mismatch repair function due to mutation in MMR genes. The initiating event in the latter is less clear but adenomas from patients with HNPCC appear to have lower rates of Wnt pathway gene mutations. It is probable that adenomas in MAP arise following somatic mutations in *APC* also, particularly given the results from my own and other investigations (Al-Tassan, Chmiel et al. 2002;

Lipton, Halford et al. 2003) . Activating beta-catenin mutations do not seem to contribute to adenoma formation in MAP or FAP although they have been found in adenomas without *APC* mutation especially in HNPCC (Morin, Sparks et al. 1997; Samowitz, Powers et al. 1999). Are there other components of Wnt signalling susceptible to adenoma-causing mutations? Although somatic mutation of *Axin2/conductin* is seen in about 10% of MSI-H colorectal cancers, it has not been documented in adenomas and may well be a by-product of defective mismatch repair rather than an initiator of dysplasia (Liu, Dong et al. 2000). Other genes such as *GSK3ß* and *Axin1* have been screened by ourselves and others both in the germline and in colorectal tumours and have been shown to have a small role if any (Webster, Rozycka et al. 2000; Lipton, Sieber et al. 2003). The question of whether other pathways or events that predict the type of APC/Wnt involvement that occurs may be involved in adenoma formation in multiple adenoma patients without germline *APC* and *MYH* mutations has not been adequately investigated. We wished to explore the contribution (or lack of it) of disruption to the Wnt/wingless pathway in these lesions.

The spectrum of any *APC* mutations in MAP adenomas and cancers is distinct from that in FAP or sporadic adenomas. We hoped to find in the mutation-negative multiple adenoma patients a predominance of different mutation types rarely seen in sporadic or FAP adenomas which might give clues regarding defects in DNA replication and repair (primarily), cell cycle regulation or control of apoptosis leading to certain candidate genes for investigation in the germline. Mutation spectrum was examined in individual patients as well in the group as a whole, as it is likely that heterogeneity will exist in any such collection of patients. An area of the *APC* gene including the mutation cluster region (MCR) was examined in 286 adenomas from 32 patients. Since patients without germline *APC* mutations require two 'hits' which may be mutations, allelic loss or transcriptional silencing of the gene for its inactivation, it is likely that in many cases mutations will occur in the MCR thus this is an appropriate initial screening strategy. Although sequencing of the entire *APC* sequence may be considered preferable, constraints exist when working with DNA derived from adenomas. It is very difficult to collect fresh frozen tissue as pathologists are required to examine a complete specimen for the presence of invasion. Also, patients classed as having multiple adenomas may develop dozens during their life in crops of two or three, and therefore at any given colonoscopy the practitioner may not recognise their phenotype. For this reason, our adenoma work is done on paraffin derived tumours. Most adenomas of average size can only

provide a small amount of DNA and this is generally enough to examine only part of a large gene such as *APC*.

Although *APC* is almost certainly a gatekeeper, *APC* mutations are not the only event to occur during the genesis and progression of adenomas. Other genes are likely to contribute at an early stage of adenoma formation although few of the changes commonly seen in colon cancers appear to be frequent in adenomas. Possibly some such changes occur in a subset of adenomas (K-ras mutations for example) and others only follow on from specific initiating events. Although it is known that BRAF mutations occur commonly in sporadic MSI-H colorectal cancer, it is rare to see MSI in sporadic adenomas. Common somatic mutations such as those in K-ras, *TP53*, *BCL-2, SMAD4* and *BAX* may occur at different stages in adenoma formation although they are only rarely uncovered. Such mutations may be present in small subsets of adenoma cells unable to be appreciated except in single crypt mutation studies. *TP53* mutations and down-regulation of apoptosis occur early in flat adenomas and cancers occurring in inflammatory bowel disease but are generally a late event in sporadic adenomas (Yamamura-Idei, Satonaka et al. 1994; Rubio and Rodensjo 1995; Suzuki, Honma et al. 2002). It is probable that at some stage of all advanced adenomas there is unbalanced proliferation and apoptosis.

Beta-catenin expression and cellular localisation were determined in a large number of adenomas using immunohistochemical staining. Aberrant nuclear beta-catenin staining may give some indication of abnormal activation of the Wnt pathway in adenomas (Munemitsu, Albert et al. 1995; Clements, Lowy et al. 2003). The oncogenic properties of Wnt/beta-catenin signaling stem from alteration in phosphorylation-dependent protein degradation and subcellular localization of beta-catenin from cell membrane to the nucleus, where it binds to T-cell factor (TCF) to form a bipartite transcription factor. The beta-catenin/TCF complex facilitates transcription of target genes that encode effectors for activation of cell proliferation and invasion and inhibition of apoptosis, leading to colorectal cancer development. The over-accumulation of beta-catenin in the nucleus leads to activation of a number of TCF-associated transcriptional pathways involved in colorectal tumourigenesis. The major known cause for such accumulation is inactivation of *APC*. Although theoretically, mutations or loss causing lack of function in other Wnt pathway genes may have the same result, few such changes have yet been uncovered. Other potential causes of dysregulation of beta-catenin expression include inhibition of PI3 kinase (PI3K) and AKT by prostaglandin and

extracellular factors including sodium butyrate (Castellone, Teramoto et al. 2005). There remains some controversy as to what conclusions can be drawn from these results. Several authors have written in support of a 'top down' progression of dysplasia within colonic crypts with dysplastic features and nuclear staining in luminal cells only (Shih, Wang et al. 2001; Jass, Whitehall et al. 2002). Others have noted that nuclear β-catenin expression is associated with increased tubular branching or crypt fission and appears predominantly in the crypt base (Kirchner and Brabletz 2000; Brabletz, Jung et al. 2001; Preston, Wong et al. 2003). It is likely that the amount and site of nuclear beta-catenin staining changes with the morphology of the tumour and the cell's state in terms of its position in the crypt, cycling and function. It is also possible that the pattern of nuclear staining – the adenoma's molecular morphology – depends to some extent on the type of *APC* mutation or other Wnt pathway disruption involved. In this study, the presence or absence of beta-catenin nuclear staining in adenomas has been correlated with the *APC* mutation status and *APC* LOH.

Evidence for the involvement of the SMAD4/TGFβ pathway and *TP53* mutations was sought using immunohistochemistry to assess levels of corresponding protein expression. Loss of SMAD4 expression occurs in most polyps from germline *SMAD4* mutation carriers, even those with pathogenic missense germline mutations. It is, in contrast, very rare to see lack of staining in polyps from patients without germline mutations in *SMAD4* (<5%) although sporadic colorectal cancers show loss of staining in 20-40% of cases (Woodford-Richens, Rowan et al. 2001; Salovaara, Roth et al. 2002). Thus immunohistochemistry for SMAD4 protein is an effective method of excluding a major role for *SMAD4* mutations in this group of patients.

TP53 mutations are identified in 50% or more of colorectal cancers. Mutations are primarily found in exons 5 to 8. The gene product TP53 is a sequence-specific transcriptional activator in the regulation of cell cycle progression and apoptosis. Thus it can be equated to a 'gatekeeper' of the cell cycle, its loss allowing cell survival in circumstances disadvantageous to the host. Most *TP53* mutations result in a protein with an abnormally long half-life, and the accumulation of protein from mutant *TP53* can be detected using immunochemical staining. *TP53* mutations are a later event than *APC* mutations in sporadic tumours, occurring during the adenoma to carcinoma transition. They are seen in adenomas at a low frequency (Ichii, Takeda et al. 1993). Hao et al showed that in adenomas with mild,

moderate, or severe dysplasia, mutation or allelic loss at *TP53* occurred in 4.8%, 16.7%, and 52.6% (Hao, Frayling et al. 2002).

6.2 Methods

6.2.1 Patient Selection

Patients were recruited by me, Dr Ian Frayling and Mrs Carole Cummings. Patients were selected from those attending the Cancer Research UK Family Cancer Clinic and the Polyposis Unit at St Mark's Hospital, Harrow. A proband from each patient's family had been examined for germline *MYH* mutations (Chapter 4) and *APC* mutations by F-SSCP of the entire gene and real-time PCR to exclude large deletions in *APC* (Table 6.1). All patients had developed five or more colorectal adenomas over their lifetime. 289 adenomas were available for study from 32 multiple adenoma patients from 32 families. Probands from families with apparent autosomal dominant and recessive inheritance as well those with no family history of colorectal adenomas and cancers were included in the study. Individuals with juvenile polyps or hyperplastic polyps only were not included.

6.2.2 DNA Extraction

Germline DNA extraction was performed by myself and Dr Emma Jaeger and tumours were microdissected and DNA extracted by myself . Germline DNA was extracted from lymphocytes or cell lines by standard methods. DNA was extracted from paraffin embedded adenoma tissue by microdissection from 10 μm slides using the corresponding H&E section as guidance. Adenoma pathology was assessed by Professor Nicholas Wright and me.

6.2.3 *APC* Mutation Detection

APC mutation detection was performed by me. F-SSCP was used to examine codons 1209 to 1604 of the *APC* gene in adenomas. The primers used are detailed in Table 4.10. This area includes the mutation cluster region. Samples were run on the ABI 3100 at 18°C and 24°C and samples showing aberrant bands were re-amplified and sequenced directly. In one patient with a number of somatic *APC* mutations in an area close to a common polymorphism,

cloning of the PCR product for this region was performed to determine the allele being lost (chapter 2).

6.2.4 *APC* LOH Analysis

LOH analysis was performed by me. For LOH analysis, the tumour DNA was PCR-amplified alongside the constitutional DNA from the patients using the 5q markers D5S346, D5S421 and D5S656, with the forward primer fluorescently labelled with FAM or HEX. For LOH, areas under the peaks (including stutter bands) were compared for all informative (heterozygous) markers. In informative cases, allelic loss was considered present if the relative ratio of normal:tumour peak areas was less than 0.5, or greater than 2. Samples appearing to have LOH had the analysis repeated and if not confirmed were reported as lacking LOH.

6.2.5 MSI Analysis

MSI analysis was performed by me. All adenomas included in the study were assessed for MSI using the mononucleotide marker BAT 26. Adenomas were said to be stable if there was no base slippage.

6.2.6 Immunohistochemistry

Immunohistochemistry was performed for p53 over-expression, aberrant (nuclear) beta-catenin expression and loss of SMAD4 protein by Miss Victoria Johnson and me. 5μm tumour sections were analysed using the M7001 (Dako), GC19220 (Transduction Laboratories) and SC7966 (Santa Cruz) antibodies respectively at 1/100 dilution (β-catenin, SMAD4) or 1/1000 dilution (p53) after microwaving the sections for 10 min. After counterstaining with haematoxylin, the slides were examined by two independent observers, Professor Nicholas Wright and me. Beta-catenin expression was scored as 0 to 4 if nuclear staining was present in 0, <10%, <25%, <50% or >50% dysplastic cells respectively. For p53, an adenoma was scored as positive if there was no staining of normal tissue and more than 20% of dysplastic nuclei were stained. Tumours were reported as negative for p53 if normal tissue showed no staining and <20% of dysplastic nuclei were stained. Tumours with

other p53 staining patterns were excluded from analysis. SMAD4 protein expression was scored as loss if there was absence of nuclear and cytoplasmic staining in dysplastic nuclei only with staining seen in normal cells.

6.3 Results

6.3.1 Patient Characteristics

The 32 patients represented a fairly diverse group (Table 6.1) with a median age at diagnosis of 50 years (mean 44, range 18-74). The majority 17/30 (57%) had not developed colorectal cancer and 22/31 (71%) had not required colectomy. The majority of these patients had a family history of colorectal cancer significant enough for them to have been offered screening colonoscopy. This is in contrast to MAP patients, the majority of whom appear to require colectomy at diagnosis due to the recessive nature of disease leading to late diagnosis with fairly extensive and advanced adenomas, possible with a higher CRC risk.. The median age at diagnosis was the mid 50's and hence considerably older than FAP patients but comparable to MAP. Polyp number ranged between 5 and 110 (median 17, mean 33). A total of 286 polyps were studied. Polyps were mostly tubular adenomas, although a number of tubulovillous adenomas and villous adenomas were also present. They ranged in size from 1 to 35 mm (median 4mm, mean 5.8mm). Hyperplastic polyps were present in some cases, but no serrated adenomas or juvenile polyps were noted. None of the patients had predominantly hyperplastic polyps. Although some individuals appeared to have a dominant inheritance of colorectal cancer, none had documented dominant inheritance of multiple adenomas. Sixteen patients had developed colorectal cancer Tissue from colorectal cancer was available in eight cases and this was examined alongside adenomas.

6.3.2 *APC* Mutation Screening.

APC codons 1209 to 1604 were screened for mutations in 247 adenomas (Table 6.2). Of these, 34 harboured probable pathogenic mutations (13%). A further nine adenomas had aberrant bands on F-SSCP but did not have detectable mutations on sequencing. As part of an ongoing study, these adenomas are undergoing more detailed analysis using cloning techniques. The majority of variants were insertions or deletions of one or more bases

causing frameshifts to occur. Nonsense (protein truncating) mutations were apparently very uncommon comprising 1/33 (33%) of mutations. Apart from the common codon 1309 mutation, del AAAGA, all frameshifts were found between codons 1430 and 1580. This is in keeping with the pattern of somatic mutations in sporadic rather than FAP tumours (Rowan, Lamlum et al. 2000). No adenoma had the common R1450X variant. The proportion of adenomas with somatic *APC* mutations found was lower than that found in another group of 156 adenomas from mutation positive FAP patients 33/247 (13%) vs. 52/156 (33%), Fisher's exact test, $p<0.01$ (Crabtree, Sieber et al. 2003). This may not be a strictly representative result as Crabtree et al used fresh frozen adenoma tissue which is likely to yield DNA of better quality with more mutations found. The proportion of adenomas with pathogenic *APC* mutations, 33/247 (13%) was not significantly lower than that for MAP patients (22/105, 21%, Fisher's exact test, $p=0.09$) (Lipton, Halford et al. 2003). The mutation spectrum was very different in the two groups, however.

Overall, 12 patients had at least one somatic *APC* mutation detected. Eighteen patients had no *APC* mutations detected in adenomas or cancers. Of these 18, twelve had five or more adenomas analysed and three had a colorectal cancer analysed. The median age of patients with *APC* mutations was 53 (mean 52) compared with a median age of 49 (mean 46) for those without mutations. Only one patient, 13, had an APC mutation in an invasive tumour. Of the patients without *APC* mutations, nuclear beta-catenin expression was seen in a proportion of adenomas in 9/18 (50%) of patients. This may imply that *APC* mutations were missed in a number of adenomas, that these adenomas had other changes such as LOH missed in this study due to poor DNA quality or epigenetic silencing leading to the dysregulation of the Wnt pathway or that beta-catenin may accumulate in the cell without loss of Wnt pathway mediated suppression.

None of the characteristic G→T transversion mutations were seen in this group of patients whilst they predominated in the MAP adenomas.

Patient two was found to have ten frameshift mutations from ten separate adenomas, eight of these in an area of only 62 codons (codon 1431-1493). There was no consistent pattern of changes otherwise. This individual was heterozygous for the common polymorphism codon 1493 G>A. It was therefore possible to clone this area in a number of adenomas to determine

whether the mutations were consistently affecting one allele, to explore the possibility of a cryptic *APC* mutation on the other allele. Somatic mutations in this area of the *APC* gene (3' to codon 1400) would typically be associated with a germline mutation 5' to codon 1280. The six polyps cloned harboured the mutations; codon 1489 ins T (two polyps), codon 1489 ins C, codon 1493 del A, codon 1472 del 55 bp and codon 1488-89 del TT (Figure 6.). At least 15 clones were sequenced for each mutation. Overall it appeared that frameshift mutations were occurring only on the G allele of the polymorphism (Table 6.2). For unknown reasons, three mutations were not seen in any clone. In two of these, the A allele predominated with the wild-type leading to a possible association of the G allele with the mutation. In one set of clones, however, no mutation was seen and the G allele predominated. F-SSCP and direct sequencing have been used to screen this patient's germline DNA for mutations in the entire coding sequence of the gene yet it remains possible that an occult mutation is present. These results are not conclusive. All of these adenomas were microsatellite stable.

Another patient (20) was found to have an identical mutation, codon 1355 del GA, in seven of his adenomas but not in another 11. As these adenomas were all taken from the same paraffin block it may imply that there were several portions of one adenoma tested. Normal tissue DNA did not contain this mutation.

When adenomas with *APC* mutations and SSCP changes were taken together, it appeared that adenoma size had a small effect only. Of the forty-three adenomas in this category, 17 were ≤5mm and 26 were >5mm.

6.3.3 Beta-catenin Immunohistochemistry

146 adenomas were successfully stained for aberrant nuclear beta-catenin expression. Of these 61/146 (41.8 %) showed no nuclear staining in dysplastic cells, 10/146 (6.8%) showed <10% of nuclei stained, 23/146 (15.7%) showed <25% of nuclei stained, 23/146 (15.7%) showed 25-50% of nuclei stained and 29/146 (19.9%) showed >50% stained (Table 6.3). Thus 58.2% of patients showed some degree of nuclear staining. This was not greatly different to the incidence seen in sporadic colorectal adenomas (Kinzler and Vogelstein 1996; Brabletz, Herrmann et al. 2000). It is also not significantly different from the rate of nuclear

staining in MAP adenomas (16/38 vs 85/146, Fisher's exact test, p=0.21) or the rate of nuclear staining in sporadic colorectal cancers (Lipton, Halford et al. 2003). Adenomas and aberrant crypt foci from FAP patients also show similarly frequent nuclear β-catenin immunostaining (Takayama, Ohi et al. 2001).

A group of nine colorectal cancers from the patient group were also stained for β-catenin. Of these, 6/9 (66%) showed no nuclear staining and three (34%) showed nuclear staining (Two had 2+ staining and one had 4+ staining). None of those without nuclear β-catenin staining in their tumour showed staining in adenomas whereas the three patients whose cancers showed nuclear staining all had a proportion of adenomas showing such staining. None of the three cancers with nuclear β-catenin staining had detectable *APC* mutations. Pathologists examining the stained adenomas felt that there was convincing evidence that β-catenin staining was generally stronger in crypts than at the luminal surface and in some cases was only present in crypt bases.

Size, more than any other variable appeared to influence the incidence and intensity of nuclear β-catenin immunostaining (table 6.4). Of the 61 adenomas of 5mm or less in diameter, 22 (36%) had some degree of nuclear staining whereas 62/85 (73%) of those over 5mm had aberrant nuclear staining (p=0.00001, Fisher's exact test). This is in keeping with data collected by Brabletz et al in sporadic adenomas. This group found that nuclear β-catenin expression was strongly correlated with adenoma size and c-myc expression but not with proliferative activity (Brabletz, Herrmann et al. 2000).

6.3.4 5q LOH and MSI Analysis

Of the 199 adenomas informative for at least one 5q marker, 43 (22%) showed LOH. The association between LOH and mutation status is shown in table 6.5. Seventy-one adenomas were not informative at any of the three 5q markers. This rate of LOH was equivalent to that found in adenomas from FAP patients with known germline mutations in *APC* (Lamlum, Papadopoulou et al. 2000). Only 5% of informative adenomas could clearly be seen to have two hits at *APC*, in all cases one mutation and one loss. A further 31% had one detectable hit, fairly evenly divided between mutation (14%) and loss (17%). Sixty-four percent of

adenomas had no detectable change at the APC locus. In this latter group 68/129 (53%) were ≤5mm and 61(47%) were >5mm in diameter. Of the adenomas with two detectable hits, five were <5mm and five were >5mm. The adenomas without evidence of *APC* changes did not appear to have an increased involvement of other pathways. Five of 31 (16%) adenomas in this category examined for p53 expression showed aberrant nuclear staining and none had loss of SMAD4 protein. 18 of 51 (35%) adenomas examined for β-catenin expression had aberrant nuclear staining, less than the group as a whole.

No adenomas were found to be microsatellite unstable at BAT 26. This is in keeping with the finding that the presence of a person in a family with more than five adenomas is negatively associated with mutation-positive HNPCC (Chapter 7).

6.3.5 SMAD4 and P53 Immunohistochemistry

Ninety-three adenomas were examined for aberrant nuclear p53 expression. Only adenomas showing nuclear staining in more than 20% of dysplastic cells were scored as positive. Overall 14/88 (16%) of adenomas had aberrant nuclear staining. This is similar to the percentage reported in sporadic adenomas. Estimates vary between 20 and 40% depending on the grade of dysplasia in adenomas (Kaklamanis, Gatter et al. 1993; Hosaka, Aoki et al. 2002). It is higher than that seen in adenomas from MAP patients 14/88 vs 2/41 (Fishers Exact test, p=0.005). Six cancers were examined and 3/6 (50%) had strong aberrant nuclear staining. Loss of SMAD4 staining did not occur in any of the 156 adenomas studied in this series with immunohistochemistry. Six cancers from persons in the study were stained for SMAD4 protein and 2/6 (33%) had lost SMAD4 expression.

6. 4 Discussion

The evidence gathered in this study appears to support the hypothesis that there may be pathways to the development of dysplasia in the colon distinct from those involving dysregulation of the Wnt and MMR pathways. I have taken a group of patients who have a multiple adenoma phenotype with no known genetic cause and hypothesise that there may be

more than one way to dysplasia than through *APC* mutation and MSI+. A subset of adenomas has been shown to exist without two detectable hits at *APC* and without accumulation of nuclear β-catenin but also without evidence of impaired DNA mismatch repair. Although using SSCP with direct sequencing some *APC* mutations may have been missed, the great majority of adenomas tested, 204/247 (83%) did not have *APC* mutations and at least 64% of those informative for both did not have *APC* LOH or mutations. Of these, changes in *TP53* and *SMAD4* were as infrequent as in the group as a whole (16% and 0% of adenomas respectively). The observation that all *APC* mutations detected were clustered in less than half of the patients leaves a substantial number for whom a different mechanism may be initiating adenoma formation. Although this is a very interesting hypothesis, several factors make it a highly guarded one. Firstly, only a proportion of *APC* was sequenced and mutations in other places could putatively play a role. Secondly the quality of paraffin derived DNA is somewhat lower than that derived from fresh frozen tissue and mutations may be missed. Thirdly, it would be narrow-minded to believe that only mutation and loss in the *APC* gene can inactivate the Wnt pathway. Events such as large genomic deletions, post translational modification of APC or epigenetic silencing as well as changes in other involved genes need to be ruled out before concluding that the Wnt pathway does not contribute to carcinogenesis.

It is possible that in some cases the Wnt pathway may be activated by mechanisms other than *APC* mutation or loss. Of the 59% of adenomas showing abnormal accumulation of β-catenin in the nucleus, only 13/85 (15%) showed *APC* LOH and 17/85 (20%) had *APC* mutations. Activating mutations in beta-catenin are one possible explanation. These have been found in small adenomas (<10mm) with a frequency of 12.5% and larger adenomas with a frequency of 2.5% and only 1.4% of invasive cancers (Samowitz, Powers et al. 1999). It is possible that the some of these adenomas are MSI-H from sporadic or inherited causes as several studies have shown an association between MSI in tumours and somatic beta-catenin mutations (Kim, Kang et al. 2003; Johnson, Volikos et al. 2005). There is apparently a strong correlation between adenoma size and Wnt pathway activation as shown by the progressive increase in aberrant nuclear beta-catenin staining with increasing adenoma size. This is supported by a study showing a strong increase in nuclear beta-catenin staining between aberrant crypt foci and adenomas from the same individual (Takayama, Ohi et al. 2001). Interestingly, there was not such a strong trend for increasing numbers of *APC* mutations with adenoma size. This lends support to the notion that *APC* mutations may not be the only

method of aberrant Wnt activation . These results as a whole imply the existence of non-APC, non-Wnt pathways in adenoma development as well as some evidence for the involvement of Wnt signalling and beta-catenin accumulation independent of *APC* mutation or loss.

Can we then derive evidence from the *APC* mutation spectrum in these adenomas as to underlying mechanisms of adenoma development? The *APC* mutation database lists some 762 somatic *APC* mutations (Béroud 2006). Of these 309 (41%) are transversion and transposition mutations. The majority of these are protein truncating (270/309, 87%) with the remaining 39/309 (13%) made up of missense amino acid changes. The remaining 453 somatic mutations on the database are frameshift mutations, insertions and deletions. In the group of adenomas reported here, there is a definite predominance of frameshift changes over truncating and non-truncating missense changes with only four of the latter found and only one definite nonsense mutation. Mutations at codon 1309 made up 6/33 (18%) of those in the cohort. This is a common mutation in sporadic tumours making up 55/761 (7.2%) of somatic mutations reported in the APC database (Béroud 2006). Frameshift changes may have been discerned more easily than missense changes in this cohort as contamination of dysplastic epithelial tissue with normal cells may have made the missense changes less easy to appreciate with smaller peaks in the sequencing read-out whereas the disruption caused by a frameshift change is more obvious. Thus the somatic mutation spectrum in this group of adenomas appears to be different from that of adenomas from *APC* mutation carriers and from sporadic adenomas developing after somatic *APC* mutations. A marked difference also exists between the APC mutation spectrum in these and MAP adenomas. The latter tend to be almost exclusively G>T transversion changes occurring at GAA motifs and causing the creation of a stop codon, with almost no frameshift mutations detected (Lipton, Halford et al. 2003).

Unfortunately the predominance of frameshift mutations in our current group of adenomas does not point specifically to any one mechanism of DNA maintenance and repair, as might an increase of certain nonsense changes (given that there is no evidence of defective mismatch repair/MSI in the tumours studied) . The lack of nonsense changes in itself is unusual although if one accepts that *APC* mutations are unnecessary for adenoma initiation and progression in these probands, the low overall number of *APC* mutations and the limited spectrum may be explained. Also, as has been previously mentioned, the use of

DNA derived from paraffin embedded tissue for mutation detection is likely to give an underestimate of true mutation frequency due to DNA quality. As many studies have used paraffin derived tissue, it would be useful to know by what extent the ability to pick up APC mutations is reduced

It is certainly possible that patients such as no. 2 with a larger number of *APC* mutations may be harbouring germline *APC* mutations or deletions which have not been uncovered with careful testing including analysis for genomic deletions. Since this study's inception, MLPA for *APC* deletions has become commonplace and we know that a number of families previously thought to be *APC* mutation negative harbour such changes. No other patient displayed such frequent mutations, yet this proband had been tested on several different occasions, and using different methods, for germline mutations. When a cloning technique was used to attempt to show segregation of the detected somatic mutations to one *APC* allele, results were not conclusive but there was some suggestion that one allele may have been consistently mutated in the soma. This gives indirect evidence of an occult germline mutation in *APC* in this proband.

In none of the adenomas tested was more than one pathogenic *APC* mutation detected. Because an area of about 400 codons only was examined, mutations in the 3' and 5' areas of the gene will invariably be missed (and colorectal adenomas rarely have the two hits in the same region of the gene, so missing at least one was almost inevitable). Our aim in concentrating on this region was to find the maximum number of mutations possible as it would be expected that a more 3' or 5' mutation or allelic loss would almost always be accompanied by a loss of function mutation in the MCR. It is difficult to obtain enough DNA from many small adenomas to perform extensive screening. One way to do so would be to obtain fresh frozen samples of adenomas. This is a difficult task given the ethics and logistics of such collection but hopefully an increased number of hospitals will persist in formalising such procedures. DNA from fresh frozen tumour material would also be more likely to allow deleterious mutations in a subset of adenoma cells to be appreciated.

Table 6.1 Clinical Characteristics of Patients

N/R = data not available

Patient	Age at dx	Polyp no.	Colon Cancer	Cancer site	FHx	Colectomy	Polyps studied	Cancer studied
1	68	28	no		recessive	Yes	9	
2	50	30	yes		none	Yes	33	1
3	19	75	no		none	Yes	25	
4	52	50	yes		none	No	2	1
5	48	24	no		none	Yes	12	
6	62	15	yes		none	No	8	1
7	70	26	yes		dominant	No	8	1
8	29	80	yes	rectum	dominant	Yes	5	1
9	36	15	yes		dominant	No	5	1
10	72	110	no		none	Yes	5	
11	56	15	no		none	No	7	
12	40	18	no		dominant	Yes	8	
13	55	10	yes	sigmoid		No	3	1
14	30	32	No		none	No	9	
15	29	>100	yes		none	Yes	6	1
16	24	18	no		dominant	No	3	
17	69	10	no		dominant	No	11	
18	18	80	no		dominant	No	8	
19	45	15	yes		dominant	No	14	
20	52	10	yes		dominant	No	18	
21	44	15	yes		dominant	No	7	
22	55	15	no		none	No	9	
23	64	>100	no		none	No	9	
24	64	60	yes	Carcinoid	none	No	19	1
25	61	20	yes		none	No	14	
26	45	15	no		dominant	No	6	
27	74	16	yes		none	No	4	1
28	62	12	yes		dominant	No	7	1
29	N/R	10	N/R			No	8	
30	N/R	8	N/R			No	1	
31	45	7	yes		dominant	No	1	1
32	62	6	no		recessive	No	2	

Table 6.2 Results of Cloning Seven Frameshift Mutations for Patient 2

Original mutation	No. of clones with polymorphism codon 1493 G>A	No. of mutations at each allele in clones.	Comment
codon 1489 ins T	A in 8 clones G in 7 clones	0 with A allele 7 with G allele	Mutation appears to occur at G allele only.
codon 1489 ins T	A in 12 clones G in 3 clones	0 with A allele 3 with G allele	Addition missense seen GAA>AAA codon 1494 in 2 clones. Mutation appears to occur at G allele.
codon 1489 ins C	A in 3 clones G in 3 clones	0 with A allele 3 with G allele	Mutation appears to occur at G allele.
codon 1493 del A	A in 13 clones G in 2 clones	0 with A allele 0 with G allele	No mutations seen but A:G ratio suggests mutation occurs at G allele.
codon 1472 del 55 bp	A in 13 clones G in 2 clones	0 with A allele 0 with G allele	No mutations seen but A:G ratio suggests mutation occurs at G allele.
codon 1488-89 del TT	A in 0 clones G in 15 clones	0 with A allele 0 with G allele	

Table 6.3 Beta-catenin Immunohistochemistry with Levels of Nuclear Staining and Association with *APC* LOH and Mutation Status

β-catenin nuclear staining		Number (%)	5q LOH (%)	*APC* mutation (%)
0	none	61/149 (40.9)	2/61 (3)	9/61 (15)
1+	<10%	10/149 (6.7)	1/10 (10)	3/10 (30)
2+	10-25%	23/149 (15.4)	3/23 (13)	3/23 (13)
3+	25-50%	23/149 (15.4)	3/23 (13)	2/23 (9)
4+	>50%	29/149 (19.4)	6/29 (21)	9/29 (31)

Table 6.4 The Association Between *APC* LOH and *APC* Mutation Status

	APC mutation +	*APC* mutation –
LOH	10/199 (5%)	27/199 (14%)
No LOH	33/199 (17%)	129/199 (64%)

Table 6.5 Correlation Between Adenoma Size and Nuclear Expression of β-catenin

	β-catenin immunohistochemistry. Level of Nuclear staining.				
Size (mm)	0	1+	2+	3+	4+
≤ 5	38	6	9	5	2
>5	23	4	14	17	27

Table 6.4 Primary data for all adenomas studied

N paraffin = normal colorectal epithelial DNA

Tissue	APC mutation	5q LOH	B-cat immuno	TP53	SMAD 4	Size (mm)	Median and mean polyps number
Patient 1							**3 and 4**
N paraffin							
adenoma 1		no	3+	0		3	
adenoma 2		no		1+		3	
adenoma 3		yes		0	no loss	2	
adenoma 4		no		0	no loss	3	
adenoma 5		no		0	no loss	2	
adenoma 6		no		0	no loss	3	
adenoma 7		no	2+		no loss	10	
adenoma 8		no	3+	0		3	
adenoma 9		yes		0		8	
Patient 2		no					**6 and 7**
N paraffin		no					
adenoma 1	SSCP change	no	0		no loss	12	
adenoma 2		no	0		no loss	13	
adenoma 3	SSCP change	no	0		no loss	8	
adenoma 4		no	0	0		2	
adenoma 5	codon 1580 del T	no	0	0		2	
adenoma 6		yes	0	1+		10	
adenoma 7	codon 1489 ins T	no	3+		no loss	6	
adenoma 8		no			no loss	8	
adenoma 9		no			no loss	6	
adenoma 10	codon 1431 del A			2+		20	
adenoma 11	codon 1489 ins C	no	1+		no loss	10	
adenoma 12	codon 1489 ins T	no	1+			10	
adenoma 13	codon 1493 del A	no	0	0		15	
adenoma 14		no	0		no loss	5	
adenoma 15	codon 1472 del 55 bp	no	0		no loss	12	
adenoma 16			2+			26	
cancer		yes	2+		no loss	cancer	
Adenoma/ cancer			2+	0		20	
adenoma 17	SSCP change	no	0		no loss	7	
adenoma 18	codon 1488-89 del TT	no	0		no loss	8	
adenoma 19		no	0		no loss	4	
adenoma 20			0		no loss	14	
adenoma 21		yes	0			9	
adenoma 22		no	0			3	

adenoma 23		no	0			3	
adenoma 24				0		2	
adenoma 25				1+		2	
adenoma 26					no loss	2	
adenoma 27					no loss	2	
adenoma 28					no loss	2	
adenoma 29					no loss	2	
adenoma 30					no loss	2	
adenoma 31			0	0		2	
adenoma 32				0		2	
adenoma 33				0		2	
Patient 3							10 and 10
N paraffin							
adenoma 1		no			no loss	11	
adenoma 2		no	4+	0	no loss	11	
adenoma 3		no	4+	0	no loss	9	
adenoma 4		no	0	0	no loss	10	
adenoma 5		no		0	no loss	9	
adenoma 6		no		0	no loss	3	
adenoma 7		no			no loss	5	
adenoma 8		no			no loss	11	
adenoma 9		yes			no loss	8	
adenoma 10		yes			no loss	8	
adenoma 11		no			no loss	6	
adenoma 12		yes			no loss	5	
adenoma 13		yes				8	
adenoma 14		NI				8	
adenoma 15		NI			no loss	11	
adenoma 16		NI				12	
adenoma 17		NI				12	
adenoma 18		NI	4+			15	
adenoma 19	codon 1309 del 5 bp	yes	4+	1+	no loss	8	
adenoma 20			4+	0		12	
adenoma 21	SSCP change		4+		no loss	13	
adenoma 22		no	4+	1+		10	
adenoma 23		no	4+		no loss	22	
adenoma 24		no	4+	0		10	
adenoma 25		no			no loss	4	
Patient 4							18 (mean)
N paraffin		no					
adenoma 1		no	2+		no loss	30	
cancer		no	2+		no loss		
adenoma 2		no		0		6	

Patient 5	L1331L, A>T nt 3993						6 and 7.5
IN paraffin							
adenoma 1		NI	0			2	
adenoma 2	1472 del C	NI			no loss	10	
adenoma 3		NI				16	
adenoma 4		NI				6	
adenoma 5		NI	3+	0		5	
adenoma 6		NI				4	
adenoma 7		NI	0	0		3	
adenoma 8		NI		0		6	
adenoma 9		NI				4	
adenoma 10		NI	2+		no loss	6	
adenoma 11		NI	2+		no loss	10	
adenoma 12	SSCP change	NI	2+		no loss	18	
Patient 6		NI					2 and 4
N paraffin		NI					
adenoma 1		NI	0		no loss	6	
adenoma 2		NI	0			15	
cancer		NI	0		no loss	cancer	
adenoma 3				0		2	
adenoma 4				0		2	
adenoma 5				0		2	
adenoma 6				0		2	
adenoma 7				0		2	
adenoma 8				0		2	
Patient 7							2 and 2
adenoma 1		no		0		2	
adenoma 2		no	0	0		2	
adenoma 3		yes				2	
adenoma 4		yes			no loss	2	
adenoma 5	C1397X	yes			no loss	2	
cancer		yes		2+			
adenoma 6				0		2	
adenoma 7				0		2	
adenoma 8				0		2	
Patient 8							5 and 8
adenoma 1		NI	2+	0		5	
adenoma 2		NI	2+	0		9	
adenoma 3		NI	0			4	
adenoma 4		NI	0			2	
cancer		NI	0	1+		cancer	
adenoma 5	codon 1556 del A	NI	2+			21	
Patient 9							5 and 5

normal							
adenoma 1		no	0			cancer	
cancer		no				4	
adenoma 2		no				7	
adenoma 3		no		0		7	
adenoma 4		no				5	
adenoma 5		no				3	
Patient 10							3 and 3
adenoma 1		no	0	0		3	
adenoma 2		no	0	0		4	
normal		no					
adenoma 3		no	0	0		3	
adenoma 4		no	0			3	
adenoma 5		no	0			3	
Patient 11		no				3	2 and 2
normal							
adenoma 1				0		2	
adenoma 2	V1404C, C>G nt4211	yes	1+			1	
adenoma 3		yes	1+			2	
adenoma 4		yes	1+			2	
adenoma 5		yes				2	
adenoma 6		yes				2	
adenoma 7		yes				2	
Patient 12							4 and 4
adenoma 1		NI	0			6	
adenoma 2		NI	0			4	
adenoma 3		NI				3	
adenoma 4		NI				3	
adenoma 5		NI	0	0		3	
adenoma 6		NI	2+	0		3	
adenoma 7		NI	2+	0		9	
adenoma 8	SSCP change	NI	2+	0		4	
Patient 13							11 and 14
adenoma 1		yes	2+			11	
adenoma 2		no	0	0		8	
cancer		no	0			cancer	
cancer		no		0		cancer	
Adenoma 3+ invasion		no	0			18	
Adenoma 4+ invasion	codon 1556 del A			1+		24	
adenoma 5		no				11	
Patient 14							3 and 3

adenoma 1		no				7	
adenoma 2		no		1+		3	
adenoma 3		no				3	
adenoma 4		no		1+		3	
adenoma 5		no				2	
adenoma 6		no				2	
adenoma 7		no				2	
adenoma 8		no				5	
adenoma 9		no				4	
Patient 15							6 and 6
adenoma 1		no					
adenoma 2		no				4	
adenoma 3		no				9	
Cancer		no		0		cancer	
cancer		no				cancer	
adenoma 4		no	0			6	
adenoma 5		no				9	
adenoma 6		no	0			6	
cancer		no				cancer	
Patient 16							9 and 8
adenoma 1		NI			no loss	10	
adenoma 2		NI			no loss	9	
adenoma 3		NI	2+		no loss	6	
Patient 17							8 and 9.5
adenoma 1		NI	3+		no loss	15	
adenoma 2		NI	3+		no loss	7	
adenoma 3		NI	3+			10	
adenoma 4		NI	3+			6	
adenoma 5		NI	3+	1+		15	
adenoma 6		NI	3+			8	
adenoma 7		NI			no loss	7	
adenoma 8		NI			no loss	8	
adenoma 9		NI			no loss	6	
adenoma 10		NI		2+		15	
adenoma 11		NI				7	
Patient 18							
adenoma 1		no	0			3	
adenoma 2		yes				3	
adenoma 3		yes			no loss	1	
adenoma 4		yes			no loss	1	
adenoma 5		yes			no loss	1	
adenoma 6		yes		0		1	
adenoma 7		yes		0		1	

adenoma 8		yes		0		1	
Patient 19							6 and 6
Normal							
adenoma 1		NI	0	0		2	
adenoma 2		NI	0	0		2	
adenoma 3		NI	4+		no loss	8	
adenoma 4		NI	4+		no loss	6	
adenoma 5		NI	4+		no loss	3	
adenoma 6		NI	4+	0		10	
adenoma 7		NI				no lesion	
adenoma 8		NI	4+	0		15	
adenoma 9		NI	3+	0		5	
adenoma 10		NI	3+	0		7	
adenoma 11		NI			no loss	1	
adenoma 12		NI	0		no loss	6	
adenoma 13		NI	0		no loss	5	
adenoma 14		NI	0		no loss	9	
Patient 20							8 and 8.5
normal						no lesion	
adenoma 1	del GA codon 1355	no	4+		no loss	6	
adenoma 2	del GA codon 1355	no	4+		no loss	10	
adenoma 3		no	4+		no loss	10	
adenoma 4		no	4+		no loss	8	
adenoma 5	del GA codon 1355	no	4+		no loss	6	
adenoma 6	del GA codon 1355	no	4+		no loss	4	
adenoma 7	del GA codon 1355	yes	4+		no loss	8	
adenoma 8		no	4+		no loss	8	
adenoma 9		yes	4+		no loss	8	
adenoma 10	del GA codon 1355	yes	4+		no loss	8	
adenoma 11		no	4+		no loss	8	
adenoma 12	del GA codon 1355	no	4+		no loss	8	
adenoma 13		no	4+		no loss	8	
adenoma 14		no	3+	1+		16	
adenoma 15		yes	3+			10	
adenoma 16		no	3+			8	
adenoma 17		no	3+			13	
Patient 21							3 and 3
adenoma 1		no	0		no loss	4	
adenoma 2		no			no loss	3	
adenoma 3		yes			no loss	3	
adenoma 4		no		0	no loss	3	
adenoma 5	SSCP change	no		0		3	
adenoma 6		no				3	

adenoma 7		no				2	
Patient 22							2 and 3
adenoma 1		no				2	
adenoma 2		no				2	
adenoma 3		yes				8	
adenoma 4	codon 1309 del 5bp	no				4	
adenoma 5	codon 1309 del 5bp	no				5	
adenoma 6		yes				1	
adenoma 7		no				1	
adenoma 8	SSCP change	no				1	
adenoma 9	codon 1560 ins C	no				1	
Patient 23							4 and 4
adenoma 1	codon 1464 del AG	no				2	
adenoma 2		yes				3	
adenoma 3		no				4	
adenoma 4		no				6	
adenoma 5		no				4	
adenoma 6		no				6	
adenoma 7		no				7	
adenoma 8		no				1	
adenoma 9		no				5	
Patient 24							4 and 5
cancer		no				cancer	
adenoma 1		no				8	
adenoma 2	T>A nt 4107, G1369G	yes				15	
adenoma 3		yes				15	
adenoma 4		no				3	
adenoma 5		no				4	
adenoma 6		no				3	
adenoma 7	codon 1309 del 5bp	yes				15	
adenoma 8	codon 1309 del 5bp	yes				3	
adenoma 9	codon 1309 del 5bp	yes				3	
adenoma 10		no				4	
adenoma 11		no				2	
adenoma 12		no				4	
adenoma 13		no				6	
adenoma 14		no				4	
adenoma 15		no				2	
adenoma 16		no				2	
adenoma 17		no				2	
adenoma 18		no				1	
Patient 25							2 and 2
adenoma 1	SSCP change	no				2	

adenoma 2		no				2	
adenoma 3	codon 1309 del 5bp	yes				2	
adenoma 4		yes				2	
adenoma 5		no				2	
adenoma 6		no				4	
adenoma 7		no				3	
adenoma 8	T>A nt 4107, G1369G	no				2	
adenoma 9		no				2	
adenoma 10		no				2	
adenoma 11		no				2	
adenoma 12		no				2	
adenoma 13		no				2	
adenoma 14		no				1	
Patient 26							3 and 3
adenoma 1		no	0	2+		4	
adenoma 2		no	0	0		3	
adenoma 3		no	0	0		2	
adenoma 4		no	0	0		2	
adenoma 5		no	0			5	
adenoma 6		no	0			4	
Patient 27							3 and 3
adenoma 1						5	
cancer			0		no loss		
adenoma 2			3+	0	no loss	3	
adenoma 3				0		2	
adenoma 4				0		3	
Patient 28							8 and 7
adenoma 1		no	0		no loss	10	
cancer		no	4+	0		cancer	
cancer		no	2+		loss	cancer	
adenoma 3		no	0		no loss	6	
adenoma 4		no	0			5	
adenoma 5		no	0		no loss	5	
adenoma 6	codon 1411 del T	no	0	0		12	
adenoma 7	codon 1411 del T	no	3+	0		11	
Patient 29							2 and 2
adenoma 1			2+			2	
adenoma 2			2+		no loss	3	
adenoma 3			2+		no loss	2	
adenoma 4			0			2	
adenoma 5			0	0		2	
adenoma 6				0		2	
adenoma 7				0		2	

adenoma 8					no loss	2	
Patient 30							3
adenoma 1	codon 1569 del 5bp, ATGAT	no	0			3	
Patient 31							6
cancer		no	0	2+			
adenoma		no	0	2+		6	
Patient 32							12
adenoma1		NI	3+		no loss	10	
adenoma2		NI	3+		no loss	15	

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Chapter 7 Refining the Amsterdam Criteria and Bethesda Guidelines – testing algorithms for the prediction of mismatch repair mutation status in the Familial Cancer Clinic

7.1 Introduction

HNPCC is currently diagnosed on molecular grounds using MMR mutation screening, aided by MSI testing of tumour DNA and immunohistochemistry for MMR proteins as outlined in chapter one. Selection of families for molecular investigation of HNPCC is usually based on imperfect methods (Amsterdam Criteria or Bethesda Guidelines – Chapter one and tables 7.1-3). Expense and resources prohibit germline mutation testing on all persons with suspicious personal or family histories. Wijnen *et al* have shown that these algorithms can be increased in sensitivity using additional clinical data in a quantitative model (Wijnen, Vasen et al. 1998). Wijnen's model, whilst appealing had not been validated in an independent data set. We wished to see whether it remained valid in these circumstances and also to see if using the detailed information we could collect regarding family history and tumour pathology we could improve on this algorithm.

A family history of CRC is one of the most common reasons for referral to the Genetics or Familial Cancer clinic. Regular surveillance, generally using colonoscopy, can greatly reduce the risk of disease (Jarvinen, Aarnio et al. 2000). However, families with apparently similar histories may have quite genetic different origins and CRC risks, including a variety of single gene disorders, 'complex' genetic inheritance or aggregation resulting from shared environment or chance. It is important to diagnose the underlying cause of familial CRC, wherever possible, because there are implications for screening regimens and risks to relatives.

One of the most common Mendelian CRC syndromes is HNPCC which results from germline mutations in DNA mismatch repair (MMR) genes, principally *MSH2, MLH1* and *MSH6* (Peltomaki 2003). HNPCC is characterised by early-onset carcinomas of the large bowel and endometrium, with more modestly increased risks of cancers of other sites (see Chapter one). Almost all colorectal and endometrial cancers and most colorectal adenomas in

HNPCC show MSI, as a result of defective MMR (Wheeler, Bodmer et al. 2000)(Loukola, Eklin et al. 2001). HNPCC tumours also usually show loss of the protein derived from the mutated gene (Lindor, Burgart et al. 2002). About 10-15% of sporadic CRCs are MSI+ owing to loss of MLH1 expression, although sporadic adenomas rarely show these features (Lindor, Burgart et al. 2002)(Loukola, Salovaara et al. 1999)(Iino, Simms et al. 2000).

Most Mendelian CRC syndromes have distinct clinical features, such as profuse polyposis, but the tumours in HNPCC cannot readily be distinguished from their sporadic counterparts. Both HNPCC and sporadic MSI+ cancers, tend to be right sided, mucinous, show tumour infiltrating lymphocytes and have a pushing margin. It is increasingly recognised, therefore, that HNPCC should be a molecular diagnosis, for example based on: (i) a pathogenic *MSH2, MLH1* or *MSH6* germline mutation; or (ii) *multiple* cancers (or a colorectal adenoma) from a family with MSI or loss of MLH1 expression; or (iii) loss of MSH2 and/or MSH6 expression in one or more of a family's tumours. Even if the causative germline mutation is cryptic, making or excluding the molecular diagnosis of HNPCC is important not only for determining risks to relatives, but also for deciding the screening protocol for those at risk (Lynch, Smyrk et al. 1995).

It is impractical to screen every case of familial bowel cancer for molecular changes suggestive of HNPCC; screening must be targeted to certain families using the clinicopathological features of the pedigree, the proband and if possible tumour pathology. The so-called Amsterdam Criteria were originally used to diagnose HNPCC if at least three family members in two or more generations had CRC, one affected person was a first degree relative of the other two, and at least one individual was diagnosed before the age of 50 (Vasen, Mecklin et al. 1991). The Amsterdam Criteria have been highly successful, with estimated sensitivity and specificity of 60% and 70% (Liu, Wahlberg et al. 2000)(Rodriguez-Bigas, Vasen et al. 1997). Certain deficiencies have, however, become increasingly recognised. For example, the original Amsterdam Criteria did not take account of extra-colonic cancers, patients with new MMR mutations were not covered, and some families with multiple polyps, but without profuse polyposis, were erroneously classed as putative HNPCC. The Amsterdam Criteria II were introduced in 1999 (Chapter one and table 7.1) and Syngal et al estimated them to have sensitivity of 78% and specificity of 61% (Syngal, Fox et al. 2000)(Vasen, Watson et al. 1999). Even these criteria however, left out a number of HNPCC associated malignancies which we now recognise.

The Bethesda Guidelines were introduced to indicate which families should proceed to MSI testing prior to screening for MMR mutations (tables 7.2-3) (Rodriguez-Bigas, Boland et al. 1997). The Bethesda Guidelines were deliberately much less restrictive than the Amsterdam Criteria. Using known MMR mutation carriers, their sensitivity has been estimated as 94%, but with specificity of only 25% (Syngal, Fox et al. 2000). Recently, modified Bethesda Guidelines were produced (Chapter one and table 7.3). The criteria for MSI or IHC testing have been broadened such that a person diagnosed at the age of 59 and with no family history of cancer may be offered testing. These criteria will probably have impressive sensitivity with very low specificity.

Wijnen *et al* set out to provide a quantitative improvement on the Amsterdam Criteria and Bethesda Guidelines by setting up a logistic regression model to predict the probability of HNPCC mutation in a given family. 184 families were recruited and all had mutation testing for *MLH1* and *MSH2*. 92 of the families met Amsterdam criteria and 47 were found to have pathogenic HNPCC mutations. All families had at least three colorectal cancers diagnosed and two thirds of families had at least one endometrial cancer diagnosed. He used the following predictive variables: mean age at diagnosis of CRC; Amsterdam Criteria-positive or -negative; number of family members with CRC; the number with endometrial cancer; presence of a patient with other cancers related to HNPCC; and presence of a patient with multiple synchronous or metachronous cancers (Wijnen, Vasen et al. 1998). In univariate analysis, factors strongly associated with mutations in HNPCC genes were younger age at diagnosis of colorectal cancer, fulfilment of the Amsterdam Criteria, a higher number of patients with colorectal cancer in a family, the presence of endometrial cancer, number with endometrial cancer, the presence of small bowel cancer, the presence of multiple colorectal cancers in one or more family members and the presence of endometrial and CRC in one person. In the multivariate analysis, mean age at diagnosis of CRC, fulfilment of the Amsterdam Criteria and the presence of endometrial cancer were the only independent risk factors.

The work of Wijnen et al established the principle of a practical, quantitative improvement on the Amsterdam Criteria and arguably the model has been under-used in clinical practice. Three potential problems remain. First, the model of Wijnen et al has not yet been verified in

an independent data set. Second, there may be good historical reasons for including a composite variable such as the Amsterdam Criteria in the regression analysis, but this may lead to unnecessarily complex input data. Third, up to 30% of HNPCC cases have germline MMR mutations which are not detected using standard techniques and a model needs to take into account HNPCC diagnosed by MSI testing and/or immunohistochemistry (Scott, McPhillips et al. 2001).

I therefore set out both to test the Amsterdam Criteria II and the Wijnen model as predictors of HNPCC which has been diagnosed or excluded using a combination of germline mutation screening, MSI analysis and immunohistochemistry. I then set up new models of our own. Finally, I verified all the models in an independent set of families. Our models have potential for targeting molecular testing for HNPCC and for allowing accurate screening advice to be given to families in which no live affected persons are available for genetic testing.

7.2 Methods

7.2.1 Selection of Families

Families were recruited by myself and Mrs Carole Cummings. Two hundred-and-fifty families were recruited from the Cancer Research UK Family Cancer Clinic, St Mark's Hospital, Harrow and from the Family Cancer Clinic, Guy's Hospital, London. Our inclusion criteria (Table 7.4) were similar to the Bethesda Guidelines. Probands from all families gave informed consent and approval was obtained form the relevant Human Ethics Committees. Families from whom archival tumour tissue specimens could not be retrieved were excluded; families without a living affected person were not specifically excluded as one of the study aims was to provide useful information to such families. Pedigree information was obtained from family members and the presence of cancer confirmed, wherever possible, from hospital records, pathology reports, cancer registry records or death certificates. Apart from distinguishing between colorectal adenomas and carcinomas, histological tumour sub-types were not taken into account when deciding whether or not to include a family. Each included family was given a score in each of 10 categories (Table 7.5).

7.2.2 Collection of Blood and Tumour Samples

Blood samples were obtained by me, Dr Ella Barclay, Dr Isis Dove-Edwin and Mrs Carole Cummings. Informed consent was obtained from patients or their next-of-kin and 10ml blood sampled where possible. Samples of all colorectal tumours and endometrial cancers from each family were requested by myself from the pathology archive at the hospital in which the operation occurred. A maximum of 3 tumours from any one family was obtained and for most families, we were able to obtain at least two tumours. In a number of families this included advanced adenomas. Tumour histology was taken from pathology reports or assessed by myself and Dr A.T. Eftekhar Sadat (pathologist) where no reports were provided. DNA was extracted from blood by standard methods. Neoplastic and normal areas were microdissected by myself from archival tissue and DNA extracted using a simple proteinase K digestion. Neoplastic tissue was marked on slides with reference to an H&E stained section in order to minimise contamination of tumour with normal tissue.

7.2.3 Mutation Analysis

Screening for *MLH1*, *MSH2* and *MSH6* mutations was performed in all families by the Kennedy Galton Centre, Northwick Park Hospital, Harrow. Where possible, we screened the person affected by colorectal or endometrial carcinoma at the youngest age in the family. In some families, mutation screening was undertaken as part of a clinical service by the Kennedy Galton Centre. Genomic DNA was amplified for each of the exons of the *MLH1* and *MSH2* genes and subjected to denaturing gradient gel electrophoresis followed by sequencing of bandshifts (Wijnen, Khan et al. 1996)(Liu, Wahlberg et al. 1998). If no mutation was found, MLPA was used for the detection of medium-sized deletions in *MSH2* (Ji and King 2001). *MSH6* was then screened if no change was found in *MSH2* or *MLH1*. Missense, truncating and deletion mutations could be uncovered using these methods, and assessed using positive control samples.

7.2.4 Microsatellite Instability Testing

All available colorectal and endometrial carcinomas and colorectal adenomas were tested for MSI by myself. MSI status was scored by looking at a combination of the mononucleotide markers BAT26 and TGFβRII and the dinucleotide markers D5S346, D18S487 and D18S46. MSI was scored if 2 or more markers or BAT26 alone showed instability in that tumour.

Although it has become standard to use Bethesda markers for MSI testing, excellent data is available supporting the use of mononucleotide repeat markers alone to distinguish MSI-H cancers (Loukola, Eklin et al. 2001)(Cravo, Lage et al. 1999)(Samowitz, Slattery et al. 1999).

7.2.5 Immunohistochemistry

Immunohistochemistry was performed by Miss Victoria Johnson and myself. Colorectal and endometrial carcinomas and colorectal adenomas were tested for loss of MLH1, MSH2 and MSH6 expression. 5μm tumour sections were analysed using the PC56 (Merc Biosciences), PC57 (Merc Biosciences) and G70220 (Becton Dickinson) antibodies respectively at 1/100 dilution after pressure cooking the sections for 4 min. After counterstaining with haematoxylin, all of the slides were examined by myself and a subset was examined by Miss Victoria Johnson and Dr Eftekhar Sadat. Protein expression was scored as negative if there was no nuclear staining in tumour areas and definite nuclear staining in normal tissue. For tumours without normal tissue, sections containing normal tissue were used on the same slide to provide a control. Sections which failed to show any staining were repeated once.

7.2.5 Determining HNPCC status

Assignment of HNPCC status was inevitably complex. I scored families as HNPCC-positive if they fulfilled one or more of the criteria shown in Table 7.6. Category 6 is justified by the facts that sporadic MSI+ cancers should all lose all MLH1 expression owing to transcriptional silencing and that there are no other known causes for sporadic MSI-H cancer. Thus, if a tumour is definitely MSI-H but MLH1 protein expression is retained, there must be a germline cause for disruption of MMR. Some such patients may have missense mutations which do not abolish protein expression but may produce a dysfunctional protein. Categories 7 and 8 take account of the fact that occasional phenocopies can occur in HNPCC families and can be justified as follows. It is estimated that 10% -15% of sporadic colorectal and endometrial cancers are MSI+ or lose MLH1 expression through transcriptional silencing and that HNPCC accounts for 2-3% of all of these cancers. It follows that a single, unselected MSI+, MLH1-absent cancer is probably sporadic, that a family with two of two MSI+ or MLH1-absent cancers is almost certainly HNPCC, a kindred with two of three MSI+ or

MLH1-absent cancers is probably HNPCC and a family with one of two MSI+ or MLH1-absent cancers is probably not HNPCC. Mutation-negative families in which one of two (or a smaller proportion) of cancers were MSI+ or MLH1-absent were therefore scored as non-HNPCC. Mutation-negative families/cases with only one cancer available and with MSI and MLH1 loss in this cancer were deemed unclassifiable and were excluded from the analysis. Category 5 is justified by the fact that sporadic adenomas show microsatellite instability in <3% of cases and sensitivity of MSI-H in an adenoma for HNPCC is 95%<.

7.2.6 Second Family Set

In order to verify each model obtained from our original 250 families, I tested it against an independent set of 94 kindreds from the Family Cancer Clinic, Oxford Regional Genetics Service. All Oxford families had undergone MMR gene mutation analysis only, as was typical at the time for many Departments of Clinical Genetics in the UK. We considered for the study all families who had undergone mutation testing and selected families using the same criteria as for the St Mark's/Guy's families (Table 7.6).

7.2.7 Statistical Methods

Statistical analysis was performed with the help of Ian Tomlinson and with advice from Richard Houlston and Mike Bradburn. STATA 7.0^{TM} was used with the family as the unit of analysis. A comparison of the features of families with and without HNPCC was carried out initially using logistic regression, and then confirmed using the χ^2 test for categorical variables and the Kruskal-Wallis test for continuous variables. Multivariate analysis was performed by logistic regression using a stepwise approach to identify independent predictors of HNPCC. The final model selected was based on the values of the pseudo R^2 and receiver-operator curves (ROCs).

7.3 Results

All 250 families from St Mark's/Guy's met the Bethesda Guidelines. One hundred-and-ten families met the Amsterdam Criteria II and 28 met the Criteria but had no affected person under 50 years of age. Thirty-eight families had a single family member affected with CRC at less than 45 years (Table 7). The 74 patients in the 'other pedigrees' group included sibships and parent-child pairs affected by CRC with one member under 45, and persons with two

CRCs and/or endometrial cancers aged over 45. Sixteen percent of families contained persons diagnosed with endometrial cancer. HNPCC was diagnosed according to our criteria (Table 7.5) in 67/250 (27%) of families. Of these, 34 had pathogenic mutations (25 in *MLH1*, eight in *MSH2* and one in *MSH6*); of the 33 mutation-negative families with HNPCC, 21 had loss of MSH2 expression and MSI+ cancers, five had absent MLH1 expression in two MSI+ cancers, two had two MSI+ cancers with loss of MLH1 expression confirmed in one of these, two had two MSI+ cancers but no immunohistochemistry data, two had one MSI+ cancer with no loss of MLH1 or MSH2 expression and one had adenomas which were MSI+. The features of patients diagnosed as HNPCC on the basis of germline mutations did not, in general, differ significantly (details not shown) from those diagnosed by MSI and/or immunohistochemistry (implying that cryptic MMR mutations have similar effects to detectable changes). The only exception was that families diagnosed by MSI and/or immunohistochemistry tended to have more individuals affected (χ^2_1=9.4, p=0.002), perhaps because a greater supply of tumours facilitated molecular diagnosis. Another reason may be that families who knew of a number of persons affected also knew details of the family history – such as the treating hospitals - better than others.

Single variable analysis (Table 7.5) showed that those in pedigree group 1 (Amsterdam Criteria II) were more likely to be HNPCC, as were families with: apparently dominant inheritance; more persons affected by CRC; lower mean age at diagnosis of CRC; low youngest age at diagnosis of colorectal or endometrial cancer; more persons with two primary tumours (colorectal or endometrial cancer); and more women with endometrial cancer. Families with five or more adenomas were less likely to be HNPCC. Number of persons affected with other cancers and the location of CRCs (right/left-sided) were not associated with HNPCC.

The Amsterdam Criteria II (category 1 families) gave 78% sensitivity, 69% specificity, 48% positive predictive value (PPV) and 71% families correctly classified in the St Mark's/Guy's families. We then attempted to improve upon the Amsterdam Criteria II using multivariate analysis. Our first observation was that no individual in category 2 (fulfilling Amsterdam Criteria II except that all affected persons were >50 years) had HNPCC by any of the definitions in table 7.5. This was an important finding for genetic testing purposes, but it inevitably led to this category being dropped from the logistic regression analysis. Categories

2, 3 and 4 were therefore combined for further analysis. We found that the Amsterdam Criteria II (variable 1/v1) were improved as a predictor of HNPCC by the incorporation into the model of: (i) v3 'number of individuals with more than one primary colorectal or endometrial cancer' (OR=2.73, 95%CI 1.37-5.42, p=0.004); (ii) v6 'mean age of CRC in the family' (OR=0.94, 95%CI 0.91-0.97, p<0.001); (iii) v4 'number of individuals with endometrial cancer' (OR=2.46, 95%CI 1.24-4.89, p=0.010); and (iv) v9 'number of individuals with 5 or more adenomas' (OR=0.24, 95%CI 0.12-0.49, p<0.001). The corresponding logit equation (Table 2) was

$$\text{Ln}(p/(1+p)) = 1.04 + 1.89\ v1 + 1.00\ v3 + 0.90\ v4 - 0.06\ v6 - 1.42\ v9$$

This "Amsterdam-plus" model was associated with pseudoR^2 of 0.35. The Hosmer-Lemeshow test showed non-significant residual variation, demonstrating a good fit of the model (χ^2_8=4.8, p=0.45).

We then determined whether a simpler "Alternative model" which did not include the Amsterdam Criteria as a variable might perform as well or better than the other models. The best fitting Alternative model resulted in use of variables v5 'number of CRCs in the family' (OR=1.39, 95%CI 1.09-1.77, p=0.009) and v7 'age of youngest with colorectal or endometrial cancer' (OR=0.93, 95%CI 0.90-0.96, p<0.001) instead of v1 and v6. The other variables in the Alternative model were: v9 'number affected with more than five adenomas' (OR=0.24, 95%CI 0.11-0.51, p<0.001); v3 'number of individuals with more than one primary colorectal or endometrial cancer' (OR=2.97, 95%CI 1.48-5.96, p=0.002) and v4 'number of individuals with endometrial cancer' (OR=3.27, 95%CI 1.75-6.13, p<0.001). The logit equation was

$$\text{Ln}(p/(1+p)) = 1.06 + 1.09\ v3 + 1.19\ v4 + 0.33\ v5 - 0.08\ v7 - 1.43\ v9$$

This model was associated with pseudoR^2 of 0.32. The Hosmer-Lemeshow goodness of fit test showed non-significant residual variation (χ^2_8=11.17, p=0.193).

Figure 7.1a shows the ROCs which demonstrate the trade-off between sensitivity and specificity in our two models and, for comparison, the Wijnen model as applied to the St

Mark's/Guy's data set. All three models performed well and similarly, with an area under the curve of about 0.86 (compared with the ideal of 1.0). For the Amsterdam-plus model, we suggest that a probability cut-off of $p>0.15$ could be used for further molecular investigation of families with suspected HNPCC; for the Alternative model, we suggest a threshold of $p>0.12$ (Figure 7.1a). For both models, these thresholds produce over 95% sensitivity, around 50% specificity and 40% PPV. Using a cut-off of $p>0.05$, the Wijnen model had similar sensitivity, specificity and PPV to our two models. These sensitivity, specificity and PPV values seem pragmatic for use in a clinical setting and the higher sensitivity compared with the Amsterdam Criteria II was gained at the expense of only modestly decreased specificity. The extra sensitivity of the quantitative models was shown when considering the fifteen true HNPCC families which did not fulfil the Amsterdam Criteria II. Using the $p>0.12$ cut-off, the Alternative model correctly predicted 13/15 families to be HNPCC. Seven of the 13 were isolated, early-onset cases of colorectal cancer, the remaining pedigrees having a predominantly early-onset colorectal cancer phenotype without an extensive family history.

The Wijnen, Amsterdam-plus and Alternative models were then verified using the patient set from Oxford which comprised twenty mutation carriers (21%). One of the Oxford families in category 2 (no-one affected under 50, but otherwise Amsterdam II-positive) had a germline MMR mutation, although no patient with 5 or more adenomas did so. The Amsterdam Criteria II performed slightly better in the Oxford data set than in the St Mark's/Guy's families, with 85% sensitivity, 62% specificity and 38% PPV. The ROCs for the three quantitative models are shown in Figure 7.1b and are similar to those obtained using the St Mark's/Guy's data set, with the exception that the Wijnen model performed slightly worse. With a $p>0.05$ cut-off, the Wijnen model gave 85% sensitivity and 40% specificity; the Amsterdam-plus and Alternative models each gave sensitivity of 90-95% and specificity of 30% using cut-offs of $p>0.15$ and $p>0.12$ respectively.

7.4 Discussion

The aims of all clinicopathological methods for predicting HNPCC are to improve diagnosis and avoid unnecessary laboratory investigations whilst not missing families who may appear to be at moderate risk only. These aims are often in conflict and the result is that no optimal model has emerged, owing to different opinions about the correct balance between sensitivity and specificity. The result is that historical or consensus criteria have predominated over

analytical models such as that of Wijnen et al (Wijnen, Vasen et al. 1998). In our opinion, a model should have a minimum of about 90-95% sensitivity and then aim to maximise specificity within that constraint. The Bethesda Guidelines, for example, have great emphasis on sensitivity. It is likely however that a significant proportion of patients tested because they fit into the modified Bethesda Criteria may be found to be MSI-H or MLH1 absent due to somatic methylation of MLH1 leading to a waste of resources in genetic testing. On the other hand, the Amsterdam Criteria II is too specific and around one third of families with germline MMR mutations will be missed.

I have tested the Wijnen HNPCC prediction model and two new models in two data sets. The conclusions are four-fold. First, I have confirmed the general finding of Wijnen *et al* that the Amsterdam Criteria II can be improved upon using quantitative approaches. Whether such approaches will prove too cumbersome for the genetic clinic remains to be seen, but the calculations proposed by Winjen and ourselves require little more than a pedigree and a calculator. Second, I have shown that the Wijnen model using a cut-off of $p>0.05$ (rather than 0.20 originally suggested) is an improvement on the Amsterdam criteria II and Bethesda Guidelines (when both of our data sets are considered). The Wijnen model also holds when HNPCC is detected using MSI analysis and immunohistochemistry in addition to mutation screening.

Third, our own models slightly out-perform the Wijnen model. Our Alternative model which does not require knowledge of or a pedigree large enough to obtain the Amsterdam Criteria also requires simpler input data than the Wijnen and Amsterdam-plus models (Table 8). The incorporation of new variables – in particular, presence of multiple colorectal polyps and number of individuals with more than one primary colorectal or endometrial cancer – can improve the prediction of HNPCC. It is also interesting that in the Alternative model, the youngest affected family member appears to be a better predictor of HNPCC than the average age affected, probably because one 'phenocopy' affected with cancer late in life can substantially distort the mean age affected within a family.

Fourth, I have shown that some aspects of the Amsterdam Criteria, such as the requirement for an individual affected at less than 50 years of age, were particularly well judged. However, the importance of other variables which have been supposed to be consistent,

independent predictors of HNPCC, particularly in the Bethesda Criteria may need re-assessment: there was, for example, no predominance of right- over left-sided colorectal cancers in our data set. Moreover, the presence of cancers apart from those of the colorectum and endometrium was not associated with HNPCC. This finding probably results from the relatively low risks of other cancers in HNPCC mutation carriers, although there may also have been some referral bias in our data set towards bowel cancer (Watson 2003).

Any of the three quantitative models could be substituted for the Amsterdam Criteria II in the family cancer clinic. Although the optimal cut-offs require refinement and may depend on the amount of mutation testing that a unit is prepared to do, all three models can give improved sensitivity (85-95%). Specificity (30-55%) and PPV (30-45%) are lower, but tolerable in a diagnostic setting and better than the Bethesda Guidelines. I suggest that all three quantitative models undergo further evaluation to assess their relative merits. If one is shown to be superior, it should be used, but if all are shown to have similar performance, then the Alternative model should be used.

In clinical practice, the family data should be ascertained and the chosen model applied. Families would be subject to further testing if an HNPCC risk greater than the threshold was predicted. Two alternative approaches can then be used. First, if the clinic has much easier access to germline mutation screening than to tumour analysis, mutation testing on the youngest available affected family member should be undertaken. If no mutation were detectable or no DNA from an affected individual were available, immunohistochemistry and MSI testing should be carried out and families classified as true HNPCC using the same diagnostic criteria which we have used above (Table 7.6). Second, if there exists ready access to local Histopathology expertise, I propose using MSI and immunohistochemistry to target mutation screening (and to diagnose HNPCC where mutations are cryptic) using the flow chart shown in Figure 7.3. The decision-making process attempts to take account of factors such as phenocopies in HNPCC families and the possibility that HNPCC adenomas might not be MSI+ which is around 50%, although high quality analysis is assumed. If the decision tree were felt to be too complex, it could be simplified by routinely performing immunohistochemical and MSI analyses together.

In summary, I have shown that quantitative models can provide better prediction of HNPCC than the Amsterdam Criteria II or the Bethesda Guidelines. In my opinion, either the existing Wijnen model or our Alternative model should be used in clinical practice, a simple computer program being sufficient to calculate risks. Families for whom the probability of HNPCC exceeds a suitable threshold can then be subject to thorough investigation using a combination of mutation screening, MSI analysis and immunohistochemistry. Using these methods, a greater number of families can be correctly classified as true HNPCC and screening can be undertaken with confidence and an appropriate risk:benefit ratio.

Table 7.1. Amsterdam Criteria II

All of the following must apply for a putative diagnosis of HNPCC to be made in a family

There are at least three relatives with an HNPCC-associated cancer (large bowel, endometrium, small bowel, ureter or renal pelvis, although not including stomach, ovary, brain, bladder or skin)
One affected person is a first degree relative of the other two
At least two successive generations are affected
At least one person was diagnosed before the age of 50
Familial adenomatous polyposis has been excluded
Tumours have been verified by pathological examination

Table 7.2. Bethesda Guidelines for MSI Testing

Tumours from any of the following should be tested for MSI (or by immunohistochemistry) and then positive cases should go forward for MMR testing.

Individuals with cancer in families that meet the Amsterdam Criteria
Individuals with two HNPCC-associated cancers, including synchronous and metachronous CRC or associated extra-colonic cancers
Individuals with CRC and a first degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma diagnosed at age <40 years
Individuals with CRC or endometrial cancer diagnosed at age <45 years
Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age <45 years;
Individuals with signet-ring-cell-type CRC diagnosed at age <45
Individuals with adenomas diagnosed at age <40 years

Table 7.3 Modified Bethesda Guidelines for MSI Testing

Tumours from any of the following should be tested for MSI (or by immunohistochemistry) and then positive cases should go forward for MMR testing.

CRC under the age of 50
synchronous or metachronous colorectal or other HNPCC-associated tumours regardless of age
CRC with 'MSI+ morphology' under age 60
CRC with one or more first-degree relatives with CRC or other HNPCC-related tumour, with one of the cancers less than age 50
CRC with two or more first- or second-degree relatives with CRC or other HNPCC-related tumour (regardless of age), including cancers (endometrial, stomach, ovarian, cervical, oesophageal, leukaemia, thyroid, bladder, ureter and renal pelvis, biliary tract, small bowel, breast, pancreas, liver, larynx, bronchus, lung, and brain (glioblastoma)), sebaceous gland adenomas and keratoacanthomas

Table 7.4 Inclusion Criteria for Families from St Mark's and Guy's Hospitals

A family (or isolated case) was included in the study if one or more of the following were fulfilled.

Amsterdam- or Amsterdam Criteria II-positive
Amsterdam- or Amsterdam Criteria II-positive, except no cancer in an individual under 50 years
One or more individuals diagnosed with CRC under 45 years
One or more individuals with two primary bowel and/or endometrial cancers
Individual(s) with one or more adenomas diagnosed under 40 years (fewer than 100 in number and having excluded germline *APC* and *MYH* mutations)
Three or more individuals affected by CRC in one generation

Table 7.5. Variables Used for Family Classification and Results of Single Variate Analysis in Predicting HNPCC Status of Family

	Variable	Odds	95% CI	χ^2_1	p
Pedigree group (relative to Group 1)	v1	6.50	3.4-12.0	39.60	<0.0001
Dominant or recessive family history	v2	1.91	1.01-3.64	4.18	0.041
No. in family with 2+ primary CRCs and/or endometrial cancers	v3	3.52	2.07-6.01	27.70	<0.0001
No. in family with endometrial cancer	v4	4.68	2.47-8.91	35.02	<0.0001
No. in family with CRC	v5	1.42	1.15-1.67	12.12	0.0005
Mean age of diagnosis in family with CRC	v6	0.95	0.93-0.96	19.20	<0.0001
Age of youngest with colorectal or endometrial cancer	v7	0.93	0.90-0.95	33.32	<0.0001
Left/right-sided CRC predominance (R≥L *v* L>R)	v8	1.81	0.93-3.30	3.11	0.083
No. in family affected with >5 adenomas	v9	0.46	0.26-0.80	12.72	0.0004
No. in family with other cancer (not CRC or endometrial cancer)	v10	1.13	0.95-1.40	1.61	0.20

Note. For v1, Group 1 = Amsterdam or Amsterdam Criteria II, Group 2 = as for Group 1 except all affecteds over 50 years, Group 3 = isolated affected person under 45 years, Group 4 = all other included pedigrees.

Table 7.6. Criteria Used for Scoring a Family or Case as HNPCC

1	Proven pathogenic *MSH2, MLH1* or *MSH6* mutation (nonsense, frameshift, or medium/large deletion)
2	Missense or putative splicing *MSH2, MLH1* or *MSH6* change previously shown to have pathogenic effects or to occur at a site of other pathogenic mutations
3	Missense or putative splicing *MSH2, MLH1* or *MSH6* change supported by MSI or loss of gene expression in that person's tumour(s)), unless that change previously been reported as polymorphism (http://www.nfdht.nl/)
4	One or more tumours with absent MSH2 and/or MSH6 protein
5	A colorectal adenoma from that kindred MSI+
6	One or more tumours MSI+ with retention of MLH1 expression
7	All cancers (minimum of two) MSI+ and/or MLH1-absent
8	Two of three cancers MSI+ and/or MLH1-absent

Table 7.7 Family Characteristics by Pedigree Group

Pedigree group		1: Modified Amsterdam	2: Modified Amsterdam, all >50 years	3: Single person <45 years	4: Other pedigrees
Total number of families		110	28	38	74
Families classed as 'HNPCC'		52 (47%)	0 (0)	7 (15%)	8 (11%)
Dominant inheritance		110 (100%)	28 (100%)	0	39 (53%)
Number of people per family with CRC	Median	3	3	1	2
	Mean	3	3	1	2
Average age at diagnosis of CRC	Median	49	64	39	55
	Mean	49	65	36	54
Age of youngest diagnosis of CRC	Median	40	57	39	49
	Mean	39	58	36	48
Families including person(s) with >5 adenomas		30/105 (29%)	11/25 (44%)	4/38 (11%)	14/69 (20%)
Endometrial cancer present		30/104 (29%)	6/25 (24%)	1/38 (3%)	3/69 (4%)

Table 7.8 Input Data Required for Each of the Models for Predicting HNPCC

Criterion	Modified Amsterdam	Wijnen	Amsterdam plus	Alternative
No. of affected persons (≥3)	X	X	X	
No. of generations affected	X	X	X	
1 affected is first degree relative of other 2	X	X	X	
1 affected person diagnosed at <50 years	X	X	X	
Mean age of affected persons		X	X	
Youngest with colorectal or endometrial cancer				X
No. with CRC				X
Any endometrial cancer		X		
No. with endometrial cancer			X	X
No. with >1 colorectal or endometrial cancer			X	X
FAP excluded	X	X		
No. with >5 adenomas			X	X

Figure 7.1 ROC Curves for the Wijnen Model, the Amsterdam-Plus Model and the Alternative Model

(a) St Mark's/Guy's data. (b) Oxford data.

(a)

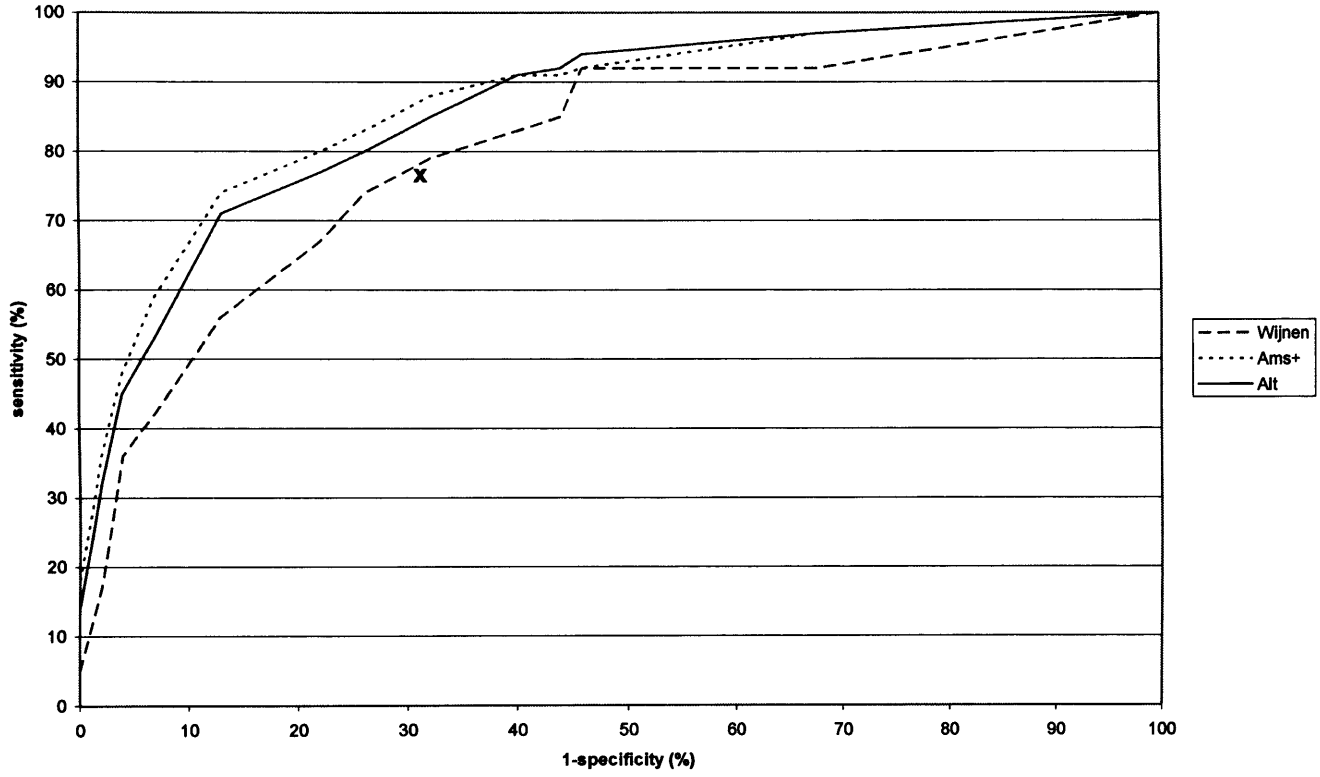
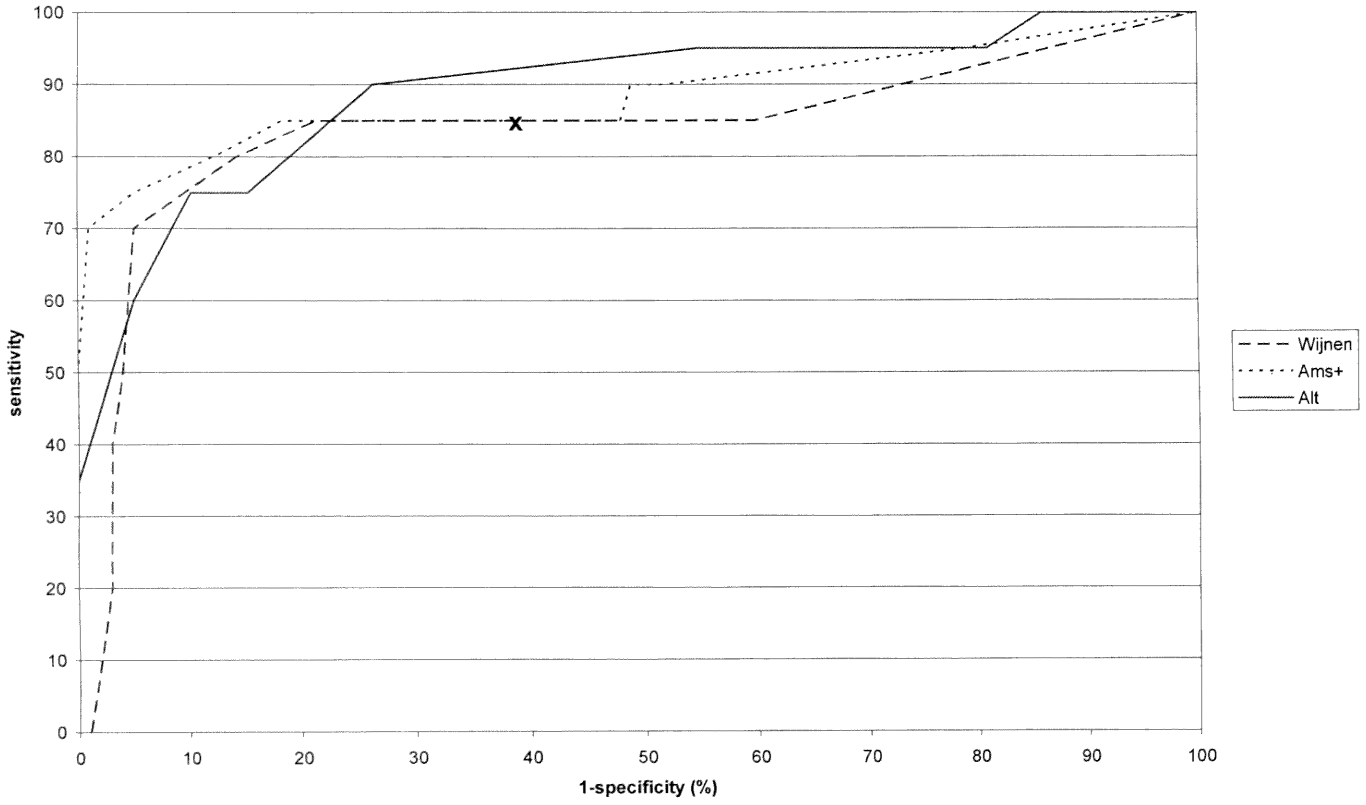


Figure 7. 1

Figure 7.1 (b)


100
90
80
70
60
50
40
30
20
10
0
sensitivity
0
10
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30
40
50
60
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90
100
1-specificity (%)
Wijnen
Ams+
Alt

Figure 7.2. Suggested flow chart for investigation and diagnosis of possible HNPCC families

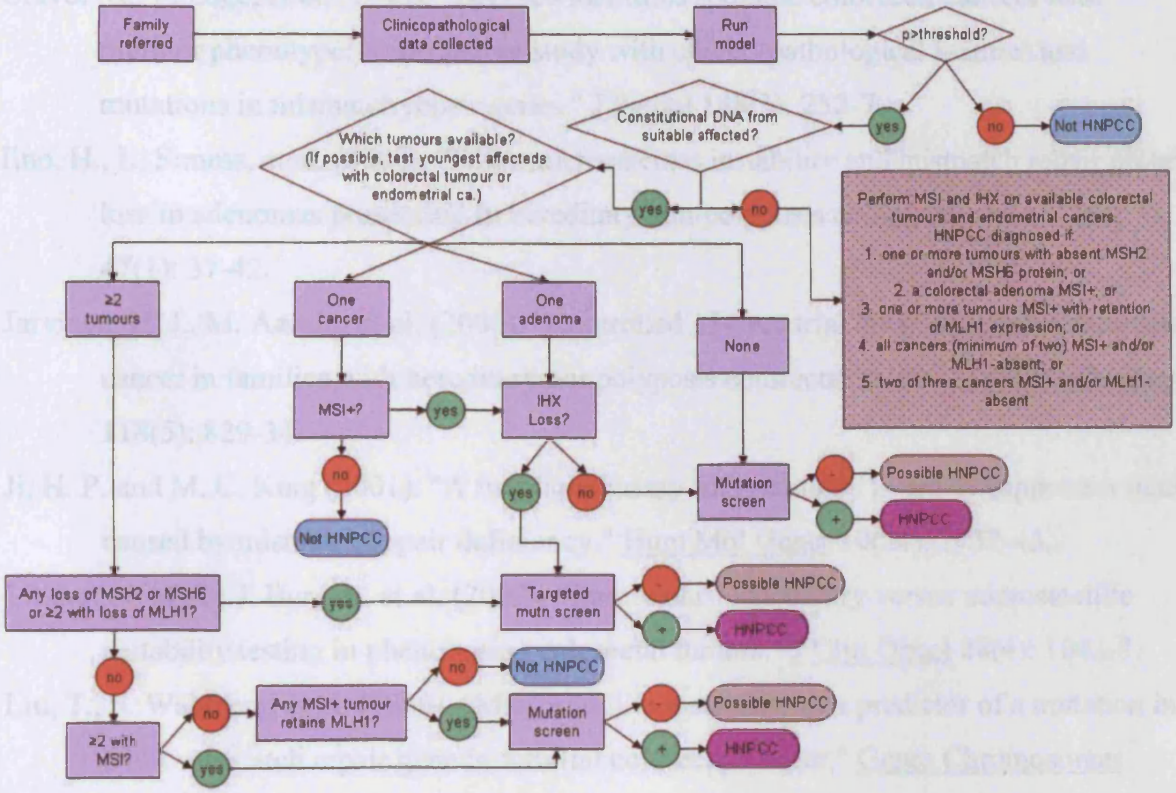

Figure 7. 2

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Chapter 8 Associations between Somatic Molecular Changes and Family History in HNPCC and Non-HNPCC Colorectal Cancer Families

8.1 Introduction

As discussed in previous chapters, epidemiological evidence suggests that about one third of all colorectal cancers have some inherited basis. About 5% of all CRCs arise as part of the known Mendelian syndromes, principally HNPCC, FAP and MAP (Kemp, Thirlwell et al. 2004). Classically, 'sporadic' colorectal adenomas arise following two *APC* mutations or LOH, with carcinoma occurring after the adenoma has progressively acquired mutation in genes such as K-ras, loss of chromosome 18q and mutation and/or over-expression of p53 (Fearon and Vogelstein 1990)(Vogelstein, Fearon et al. 1988). Some sporadic CRCs develop along a different pathway in which *BRAF* rather than K-ras mutations occur, there is a lower frequency of *TP53* mutations and a higher frequency of *BAX* mutations, the karyotype is near-diploid, and deficient DNA mismatch repair due silencing of *MLH1* due to promoter methylation renders the tumour microsatellite-unstable (MSI+) (Kambara, Simms et al. 2004)(Jass, Walsh et al. 2002).

FAP tumours appear to develop along similar genetic pathways to sporadic CRCs, probably because bi-allelic *APC* mutations are the initiating events in both cases, although FAP tumours have certain distinguishing features, such as a low frequency of K-ras mutations (Miyaki, Seki et al. 1990)(Miyaki, Konishi et al. 1994)(Lamlum, Papadopoulou et al. 2000). Tumourigenesis in HNPCC follows a pathway similar to MSI+ sporadic CRCs, although in some HNPCC cancers *beta-catenin* mutations substitute for *APC* mutations and HNPCC tumours generally have K-ras rather than *BRAF* mutations (Deng, Bell et al. 2004)(Johnson, Volikos et al. 2005). MAP CRCs follow another distinct pathway, being near-diploid and microsatellite-stable (MSI-), with a high frequency of *APC* mutations (but a low frequency of allelic loss), and a high frequency of K-ras mutation (Lipton, Halford et al. 2003). In general, the genetic pathways of HNPCC and MAP carcinogenesis reflect the underlying genetic instability specific to each condition, resulting from deficient DNA mismatch repair (MMR) and base excision repair (BER) respectively.

The differences between the genetic pathways of carcinogenesis in FAP, HNPCC, MAP and sporadic CRCs partly reflect three factors. First, owing to their DNA sequence, some genes are susceptible to certain types of mutation; thus, the 10 bp repetitive oligoadenine tract in

TGFBR2 is prone to mismatch slippage in HNPCC and these mutations tend to occur rather than the *SMAD4/SMAD2* mutations seen in MSI- CRCs, even though the former might provide a greater functional advantage (Markowitz, Wang et al. 1995)(Woodford-Richens, Rowan et al. 2001). Second, there is probably selection against co-occurrence of certain changes in CRCs; for example, a high frequency of allelic loss or aneuploidy/polyploidy rarely occurs together with defective MMR or BER, BRAF mutations appear mutually exclusive with K-ras and *APC* and *beta-catenin* mutations do not tend to co-exist within cancers (Jones, Douglas et al. 2005)(Deng, Bell et al. 2004)(Oliveira, Westra et al. 2004)(Rajagopalan, Bardelli et al. 2002). For example, in HNPCC tumours it appears that the higher incidence of *beta-catenin* mutations makes up for a lower incidence of *APC* changes (Miyaki, Iijima et al. 1999). Third, some changes - for example, the missense beta-catenin mutations in HNPCC - are probably co-selected with other mutations which have been driven by the underlying genetic instability (Johnson, Volikos et al. 2005).

The remaining 25-30% of CRCs with an inherited basis probably result from unknown moderate-penetrance and/or low-penetrance predisposition alleles. Heterozygosity for *MYH* mutation might certainly be one such low penetrance allele as may missense changes such as I1307K in *APC* (Sieber, Lipton et al. 2003)(Kambara, Whitehall et al. 2004)(Jenkins, Croitoru et al. 2006). Clusters of low penetrance genes may also be responsible. Very little is known about the somatic genetic pathways followed by cancers from high risk families outside the known Mendelian syndromes. Lindor et al undertook a study of 160 Amsterdam positive families classified for MSI+ or MSI- cancers and assessed cancer risk in relatives (Lindor, Rabe et al. 2005). Whilst the risk for relatives in MSI+ HNPCC families was as high as expected, it was markedly lower for families without MSI- cancers, particularly for colorectal and endometrial cancers. Mean age of cancer incidence amongst relatives was also much lower in relatives from MSI+ cancer families (48 versus 60 years). This suggests that Amsterdam positive families without MMR deficient cancers may have a very different genetic make-up and may be due to cluster of environmental exposure, moderate penetrance alleles or even chance alone.

In a large series of sporadic CRCs, no association was seen between family history, K-ras mutation status or MSI, but a weak association was seen between TP53 mutation status and a family history of CRC (Slattery, Curtin et al. 2002). Abdel Rahman et al have noted that aberrant nuclear beta-catenin expression and mutations are associated with cancers from

HNPCC families with a paucity of nuclear beta-catenin staining and *TP53* mutations in the MMR mutation negative cancers (Abdel-Rahman, Ollikainen et al. 2005). This hints at novel pathways of tumourigenesis in these families. If some of these remaining inherited CRCs arose as a result of defective DNA repair, it would be expected – by comparison with HNPCC and MAP – that they would tend to have ploidy, mutation spectra and frequencies of allelic loss which reflected this fact. It would be predicted, for example, that in DNA repair-deficient CRCs, allelic loss at *SMAD4* on 18q and at *APC* would be reduced in frequency, beta-catenin and K-ras mutations might be more frequent, p53 mutations would be less frequent and CRCs would tend to be near-diploid. If, however, some of the remaining inherited CRCs resulted from moderate-penetrance mutations in genes more similar to *APC*, genetic pathways of carcinogenesis would be similar to the sporadic, unselected CRC. It is also likely that CRCs resulting from genes with low penetrance (or CRCs which were present in chance familial clusters) would follow genetic pathways which could not readily be distinguished from unselected cases. It might be possible, moreover, to distinguish different groups of CRC families of unknown genetic origin, based on differences between their pedigrees, pathological and/or molecular features.

In this study, I have analysed CRCs from five series of patients/families: (i) HNPCC; (ii) MAP; (iii) patients with multiple colorectal adenomas, but without MAP or attenuated or classical FAP; (iv) patients presenting to a Family Cancer Clinic with evidence of inherited disease, but without a molecular diagnosis of HNPCC, MAP or FAP (FCC patients); and (v) a consecutive series of CRC cases with no information about their family history. Colleagues and I have screened the CRCs for mutations in K-ras and *beta-catenin*, for MSI, for allelic loss at *APC* and on chromosome 18q (close to the *SMAD4, SMAD2* and *DCC* loci), and for the expression of beta-catenin and p53 proteins. We have compared the molecular findings with clinical, pedigree and pathological data, and tested for evidence that the FCC cases can be clustered or stratified on the basis of molecular features into more than one group.

8.2 Methods

8.2.1 Patient Ascertainment

CRC cases were derived from those referred to the Cancer Research UK Family Cancer Clinic, St Mark's Hospital and the Family Cancer Clinic, Guy's Hospital. National research ethics guidelines were followed. Recruitment was performed by Dr Ian Frayling, Dr Emma Barclay, Mrs Carole Cummings and me. A variety of families was incorporated into the study, using inclusion criteria similar to the 'Bethesda Guidelines', specifically: (i) Amsterdam or modified Amsterdam Criteria; (ii) Amsterdam or modified Amsterdam criteria in all respects except no cancer occurring in an individual under 50 years; (iii) one or more individuals diagnosed with CRC under 45 years; (iv) one or more individuals with two HNPCC cancers (CRC or endometrial); (v) >5 adenomas to date in any individual (with fewer than 100 adenomas in total and excluding germline *APC* mutations); or (vi) three or more individuals affected by CRC in one generation (Umar, Boland et al. 2004). Families from whom archival tumour tissue specimens could not be retrieved were excluded, although families without a living affected person were not automatically excluded. Pedigree information was obtained from family members and the presence of cancer confirmed, wherever possible, from hospital records, pathology reports, cancer registry records or death certificates.

8.2.2 Family Variables

Each included family was given a score in each of the following binary categories: (i) the apparent mode of inheritance - dominant or recessive; (ii) one or more persons with two primary tumours (colorectal and/or endometrial cancer) in the family; (iii) presence or absence of a woman with endometrial cancer in the family; (iv) the mean age at diagnosis of CRC (≤45 or >45 years); (v) the age at diagnosis of the youngest person with CRC or endometrial cancer (≤45 or >45 years); (vi) predominance of right- or left-sided colorectal cancers in the family; and (vii) presence or absence of a patient with five or more adenomas in the family (Table 8.1). Binary coding was necessary for statistical analysis in this project. In addition, the number of persons affected by CRC in the family and the total number of other cancers (apart from colorectal or endometrial) developed by family members were noted.

8.2.3 Collection of Blood and Tumour Samples from Families

Blood collection was performed by Mrs Carole Cummings, Dr Ella Barclay and me. Microdissection and DNA extraction were performed by Dr Halford for the cancers without known family history and by me for others. Living, affected family members were asked to give a 10ml blood sample and consent for access to archival tumour tissue. Where affected family members were deceased, their next-of-kin was asked to give consent for access to archival tumour and normal tissue specimens. This project was approved by the human ethics committees at Northwick Park Hospital, Harrow and Guy's Hospital. All available colorectal carcinomas were obtained from affected family members. The histological features of the tumours, including size, grade, mucinous component and stage, were taken from pathology reports or assessed by Dr A.T. Eftekhar Sadat and me where no reports were provided. Microdissection of neoplastic and normal areas from paraffin-embedded archival tissue was guided by reference to haematoxylin and eosin-stained sections; tumour and normal DNAs were then extracted using a simple proteinase K digestion.

8.2.4 Germline Mutation Detection and Family Classification

Molecular and immunochemical diagnosis of tumours was performed by Miss Victoria Johnson and me. *MYH* mutation testing was performed by Dr Oliver Sieber and me. *APC* mutation screening was performed by Dr Emma Jaeger and Miss Victoria Johnson. From the families ascertained from the Family Cancer Clinics of St Mark's and Guy's Hospitals, we sub-divided cases into those with germline mismatch repair mutations (HNPCC), bi-allelic *MYH* mutations (MAP), *APC* mutations (AFAP) or no mutation detectable in these genes. Molecular diagnosis of HNPCC was undertaken using the criteria in Chapter 7 which are based on a combination of mutation screening (*MLH1, MSH2* and *MSH6*) and tumour analysis (MSI testing and immunohistochemistry for MLH1, MSH2 and MSH6) (Lipton, Johnson et al. 2004). *MYH* screening was undertaken as described in Chapter 4 (Sieber, Lipton et al. 2003). *APC* screening used a fluorescence-SSCP method adapted from described protocols (Chapter 2), although no case was actually found to be *APC*-mutant (Groden, Gelbert et al. 1993). The remaining families/cases were classed as 'Multiple adenoma' (five or more proven colorectal adenomas to date) or FCC families/cases (all others).

8.2.5 Unselected Series of Colorectal Carcinomas

Analysis of this group was performed by Mr Andrew Rowan and Dr Sarah Halford. An unselected series of 100 fresh-frozen CRCs and paired normal bowel was obtained from St Mark's Hospital, London; fixed tissue was obtained from the same tumours. All cancers contained more than 60% neoplastic cells, as assessed using routine histology. Clinico-pathological data were obtained from hospital records. DNA was extracted from each tumour sample and paired normal colon epithelium using standard methods. These samples were studied on an anonymous basis according to local research ethics guidelines and their features have previously been reported by Rowan *et al* (Rowan, Halford et al. 2005).

8.2.6 Immunohistochemistry for beta-catenin and p53

Available paraffin embedded colorectal cancers were tested by immunohistochemistry for over-expression of beta-catenin and p53. 5μm tumour sections were analysed using the beta-catenin mouse monoclonal antibody (Sc 7963) from Santa Cruz Biotechnology and p53 mouse antibody (M 7001) from Dako antibodies, respectively at 1/100 dilution after pressure cooking the sections for four minutes. After counterstaining with haematoxylin, the slides were examined by three independent observers, Dr A.T. Eftekhar Sadat, Miss Victoria Johnson and me. For beta-catenin, nuclear expression was scored as positive if more than 5% of cells had nuclear staining and negative otherwise. For tumours without normal tissue, sections containing some normal tissue were used to provide an internal control in the case of beta-catenin. For p53, a cancer was scored as positive if there was no staining of normal tissue and more than 20% of nuclei were stained. Tumours were reported as negative for p53 if normal tissue showed no staining and <20% of neoplastic nuclei were stained. Tumours with other p53 staining patterns were excluded from analysis. For p53 controls, tumours known to be positive for nuclear staining were used.

8.2.7 Mutation Screening for K-ras and beta-catenin

Mutations in K-ras (codons 12, 13 and 61) and *beta-catenin* (exon 3) were detected in each cancer using direct sequencing in forward and reverse orientations as previously described (Chapters 5 and 7). This was performed by Victoria Johnson, Emmanuel Volikos and me.

8.2.8 Allelic Loss (LOH) Analysis

LOH was assessed at the *APC* and *SMAD4* loci (the latter of these also referred to as 18q LOH). Microsatellites very close to each locus ((D5S346 and D5S421 for *APC*; D17S487, D18S46, D18S474 and D18S35 for *SMAD4*) were typed in each cancer and in a sample of constitutional DNA. Constitutionally homozygous markers or markers showing MSI in tumours were scored as non-informative. Otherwise, at each marker, LOH was considered to be present if the area under one allelic peak in the tumour was less than 0.5x or greater than 2x that of the other allele, after correcting for the relative allelic areas using the constitutional DNA. If there was any discordance among markers, the marker(s) closest to the gene of interest were given precedence in classifying the cancer.

8.2.9 Data Scoring

In order to make analysis practicable, we scored each of the molecular variables as a single binary data point for each family or isolated case. If it were only possible to analyse a single cancer for that family, those results were used. However, we reasoned that where multiple cancers were available, we should obtain as much data as possible in order to reduce the effects of chance variation in genetic pathways, and then assess each family/case on the basis of all available cancers. For families/cases in which more than one cancer was analysed, we therefore scored molecular changes according to the most frequent result; for example, if two of three cancers had a particular change, that family was scored as positive for that change but if one of three cancers had the change, the family was classed as negative. If the same number of cancers was with and without the change, we classed the family/case as positive.

8.2.10 Statistical Analysis

Single variable tests (Fisher's exact, and Wilcoxon) and multi-variable analysis (logistic regression) were performed using STATA 7.0. We performed hierarchical cluster analysis

using the *cluster averagelinkage* command of STATA. Occasional PCR failures or exhaustion of samples meant that not every data point was available for each of the tumours; missing data points for the cluster analysis were substituted by the mean across the whole patient set. The *cluster generate* command of STATA with two groups was used to partition out clustered groups of cases/families. Where testing specific hypotheses or confirming previously reported associations, or in the multivariable analysis, we used a threshold of p=0.05 to indicate statistical significance. Where searching for new associations in a single variable analysis we used the more restrictive threshold of p=0.01 in order to make allowance for multiple testing.

8.3 Results

I initially examined the frequency of each molecular change in the five series of patients/families (Table 8.2). K-ras mutations were found at similar frequencies (0.27-0.34) in each of the series, except for the MAP patients (0.64); the K-ras mutation frequency in the MAP carcinomas was significantly higher than in the other Multiple adenoma patients (p=0.009, Fisher's exact test) and higher than in all the other patient groups combined (p=0.01, Fisher's exact test). The frequency of LOH at *APC* ranged from zero in the MAP cancers to 0.36 in HNPCC and 0.58 in the multiple adenoma patients; with the exception of MAP, there were no significant differences in *APC* LOH frequency among the groups (p>0.14 in all cases, Fisher's exact test). The frequencies of 18q LOH ranged from 0.36 in HNPCC to 0.55 in the FCC patient group ; the only significant difference between 18q LOH frequencies was when comparing these two groups (p=0.043, Fisher's exact test). adenomas and MAP cancers also had 18q loss in around 50% of cases. As would be expected, MSI was present in all HNPCC cancers and none of the MAP cancers. The frequency of MSI+ was not statistically different in FCC patients (13%) to that in unselected patients (10%). All multiple adenoma cancers were MSI-. Beta-catenin mutations were almost exclusively found in the HNPCC cancers, as previously reported, although nuclear expression of beta-catenin protein was actually less frequent in these cancers than others (although not significantly so) (Johnson, Volikos et al. 2005). p53 over-expression was fairly constant throughout all groups with over 50% of all tumour types showing staining.

I then focussed on the FCC patient series. I performed searches for pairwise associations between the molecular variables and for associations between the molecular and

clinicopathological variables. The only association detected between the molecular variables was between LOH at *APC* and LOH on chromosome 18q (p=0.004, Fisher's exact test; Table 8.2). This observation may reflect the common co-occurrence of these changes in aneuploid/polyploid lesions (Jones, Douglas et al. 2005). Logistic regression analysis confirmed the association between *APC* LOH and 18q LOH, but did not reveal further associations between the molecular variables.

K-ras mutation, *APC* LOH, 18q LOH and nuclear beta-catenin expression were not associated with any of the clinico-pathological variables in the FCC patient series. Absence of p53 over-expression was, however, associated with a lower age of the youngest person with colorectal or endometrial cancer in the family (Fisher's exact test, p=0.0012). For those families with p53 over-expression, mean age of the youngest affected individual was 37 (median=39, interquartile range=29-43); for those without, mean age of the youngest affected individual was 47 (median=46, interquartile range=40-56). Multi-variable analysis did not reveal any additional associations between the molecular and clinico-pathological data.

Cluster analysis was then used in order to suggest groups of FCC patients who might have similar clinical features and hence arise from different genetic origins. The 149 FCC patients were divided into four groups of 37 or 38 at random. Hierarchical cluster analysis by clinicopathological features in each group revealed no consistent clusters (details not shown). Cluster analysis using the five molecular variables (K-ras mutation, *APC* and 18q LOH, beta-catenin expression and p53 expression) was also performed. Following clustering, for each of the four cluster replicates, the families/cases were partitioned into two dissimilar groups, with the aim of identifying molecular factors which consistently discriminated between different types of patient (Figure 8.1). In all of the four cluster replicates, presence or absence of K-ras mutation was a highly significant discriminant ($p<0.002$ in all cases, Fisher's exact test, excluding missing data points). None of the other molecular variables was discriminatory in more than one of the four replicates. Only five families or patients failed to cluster correctly by K-ras status.

Cluster analysis was then performed on the multiple adenoma series of patients. Molecular data was generally similar to that in the FCC series. LOH at APC and chromosome 18q was more strongly associated overall (p=0.001, Fisher's exact test). Cluster analysis on the entire multiple adenoma series using the five molecular variables showed no consistant

discriminator when the families or cases were partitioned into two dissimilar groups. However, on partitioning into three groups, presence or absence of K-ras mutation was a perfect discriminant, all nine tumours with K-ras mutations being in groups 2 and 3 and all non-mutant tumours in group 1 (figure 8.2). None of the other molecular variables were discriminatory.

8.4 Discussion

Mutations in the germline which predispose to colorectal cancers such as *APC* in FAP, MMR genes in HNPCC and *MYH* in MAP cause tumours to develop in different ways. The tumours in each condition have fairly uniform genetic profiles and in some cases, histology. The underlying genetic defect guides the pattern of somatic changes seen in the tumour. This has been demonstrated in FAP, HNPCC and more recently by me in MAP (Chapter 5). In this study I attempted to use molecular data from tumours as well as clinico-pathological variables to stratify FCC patients and families into strata for further investigation.

One obvious comparison is between cancers arising in the setting of MAP and those from patients with MA and no known genetic cause. In the groups studied, MAP cancers had molecular features which were different from those of the multiple adenoma patients. K-ras mutations were particularly frequent in MAP as previously documented and, specifically, more common than in the multiple adenoma cases (Lipton, Halford et al. 2003). *APC* LOH was infrequent in MAP compared with other cancers, including those from multiple adenoma patients. This may well be because the primary method of *APC* inactivation in MAP tumours is through G→T transversion mutations which are known to occur commonly in MAP due to defective BER. MSI was absent in tumours from both MAP and Multiple adenoma patients. This result was not unexpected as BER and MMR deficiency are unlikely to be a viable combination in MAP cancers and the multiple adenoma phenotype is negatively correlated with HNPCC (Lipton, Johnson et al. 2004). My results suggest that multiple adenoma patients without germline mutations in *MYH* or *APC* do not result from a mutator phenotype at the base pair level which is comparable with that of MAP or from a defect in MMR.

I found that p53 over-expression was equally common in HNPCC, multiple adenoma and FCC cancers (68-72%). The level of expression found in HNPCC cancers was high given

that there is frequent inactivation of the p53 pathway in such cancers due to mutations in *BAX* fulfilling a similar role (Percesepe, Pedroni et al. 2000)(Yagi, Akiyama et al. 1998). It would be an interesting further project to check for BAX mutations in the HNPCC subset of cancers in this study. As others have found, the frequencies of LOH at *APC* and on chromosome 18q were lower in HNPCC carcinomas than in the other series, although these differences did not reach statistical significance and could not reliably be used to distinguish HNPCC patients from the FCC cases or Unselected series (Johnson, Lipton et al. 2005)(Konishi, Kikuchi-Yanoshita et al. 1996). Generally lower frequencies of allelic loss are seen in MSI+ cancers, both sporadic and inherited possibly because of lack of viability of cells will chromosomal instability and MMR defects (MSI+CIN+).

In this series the frequency of MSI+ in FCC cases was approximately the same as for unselected and multiple adenoma cancers. These data suggest that the previously used strategies for detection of HNPCC - immunohistochemistry for MLH1, MSH2 and MSH6 as well as MSI on as many tumours as are available in a family, followed by mutation testing of *MLH1, MSH2* and *MSH6* as indicated - have been highly effective in 'weeding out' those with a germline mutation (Lipton, Johnson et al. 2004). It may have been expected that, if all HNPCC had been extracted from the FCC group, that the prevalence of MSI+ cancers would have been lower than that in the unselected group as the remainder should be associated with somatic epigenetic mutation which increases with age and the average age of sporadic cancer patients was 67 as compared to 51 in the FCC group. The reason that this was not the case may relate to only a phenocopy tumour being available in the family for testing or one family member's tumour with loss of MLH1 and MSI+ who is not available for mutation testing with no other tumours available. The latter would have been classified as non-HNPCC if the person was >45. The hypothesis that more HNPCC remains to be detected in the FCC group is supported by the strong association of absent p53 over-expression in the FCC patients with a family member affected at a young age. Alternative, but less plausible, explanations for these data are germline mutations at unknown loci involved in mismatch repair and unknown germline variation which predisposes cancers in these patients to follow a mismatch repair-deficient pathway. Based on the 13% frequency of MSI in the FCC series compared with 10% in the unselected CRCs, perhaps 3-5% of FCC patients were actually affected by HNPCC in this study.

The similar frequencies of nuclear beta-catenin expression in the different CRC series and the absence of an association between nuclear beta-catenin and any clinico-pathological variable suggest that some form of Wnt pathway dysregulation may be common to all types of CRC, even if not all tumours achieve this in the same way. The cases without nuclear beta-catenin localisation may be developing along a different pathway or may represent tumours with more subtle Wnt dysregulation. Other explanations include production of a variant beta-catenin protein which does not react with the staining antibody or dimerisation or interaction with other proteins which abolishes an antibody binding site. Previous reports that not all FAP carcinomas show nuclear beta-catenin, despite obligate bi-allelic *APC* mutation, suggest that the latter is at least partly true (Kobayashi, Honma et al. 2000).

The fact that the FCC series was consistently divided into two groups by presence or absence of K-ras mutation suggests that this may be a true classifier of familial bowel cancers of different, but unknown genetic origins. Under-diagnosis of HNPCC cannot explain this classification, since K-ras mutation and MSI were not associated in the FCC cancers. There is precedence for a classification of CRCs based partly on K-ras status, given the relatively high K-ras mutation in MAP and the low frequencies in FAP and sporadic and hereditary, MSI+ CRCs, even though the functional basis of these observations is not well understood. It is interesting to note that in comparison to MAP cancers which are predominantly left sided, in a group of over 300 sporadic adenomas, those in the rectum contained more k-ras mutations (Barry, Baron et al. 2006). Variable reports are given for K-ras mutations in HNPCC tumours with most investigators finding low numbers of mutations compatible with our series (Losi, Ponz de Leon et al. 1997)(Young, Simms et al. 2001). Sporadic CRC and adenomas on the other hand tend to show high levels of K-ras mutation. K-ras is certainly central to cellular events in colorectal cancer, showing interactions with the Wnt pathway through GSK3ß and TCF as well as effects on VEGF and interaction with the BRAF(Li, Mizukami et al. 2005).

In summary, there is considerable overlap between the genetic pathways followed by colorectal cancers in the known dominant and recessive inherited syndromes, in undiagnosed families and cases from the Family Cancer Clinic and in unselected CRC patients. Specific differences between these pathways are also well established, although they cannot currently be fully explained. I have identified a set of patients with multiple adenomas and no evidence of (attenuated) FAP or MAP, and these patients' tumours resemble unselected CRCs and FAP CRC's more than MAP CRCs. There is additional evidence to show that a

minority of the remaining CRC kindreds from family cancer clinics may be true cases of HNPCC, with cryptic mutations and a family history which makes tumour-based diagnosis difficult. Nevertheless, it is possible that K-ras mutation status distinguishes two groups of non-HNPCC, FCC patients who currently have unknown genetic origins.

Table 8.1 Clinical Features of the Cases and Families Studied

Group (No. of cancers	Dominant inheritance	>1 primary cancer	Endometrial cancer present	Mean age <45 years	Youngest age <45 years	R≥L	Any patient with ≥5 adenomas	Mean no. of persons with colorectal cancer (range)	No. of persons with cancer other than colorectal or endometrial
HNPCC (43)	34/43 (82%)	20/42 (48%)	18/42 (43%)	20/42 (48%)	29/42 (69%)	27/40 (68%)	3/42 (7%)	2.5 (1 to 9)	1 (0 to 6)
MAP (17)	8/17 (47%)	3/17 (18%)	0/17 (0%)	2/17 (12%)	2/17 (12%)	12/17 (71%)	17/17 (100%)	1 (0 to 3)	0 (0 to 4)
Multiads (49)	38/47 (81%)	10/47 (21%)	2/47 (4%)	9/46 (20%)	17/46 (37%)	18/32 (56%)	49/49 (100%)	2 (1 to 6)	0 (0 to 4)
FCC (150)	81/137 (59%)	18/117 (15%)	11/116 (9%)	37/119 (31%)	66/117 (56%)	57/101 (56%)	0/150 (0%)	2 (1 to 6)	0.5 (0 to 6)

Table 8.2 Numbers of Families/Cases Studied from Each of the Five Series and the Frequency of Each Molecular Change

Series (no. cancers)	K-ras	*APC* LOH	18q LOH	MSI	Beta-cat mutation	Beta-cat IHC	p53 IHC
HNPCC (43)	11/39 (28%)	10/28 (36%)	9/25 (36%)	43/43 (100%)	5/34 (15%)	17/37 (46%)	23/32 (72%)
MAP (17)	9/14 (64%)	0/13 (0%)	7/14 (50%)	0/17 (0%)	0/16 (0%)	12/17 (71%)	8/15 (53%)
Multiads (44)	11/41 (27%)	18/31 (58%)	15/28 (54%)	5/49 (10%)	N/D	27/45 (60%)	28/39 (72%)
Familial CRC (149)	34/117 (29%)	54/112 (48%)	47/86 (55%)	18/139 (13%)	0/54 (0%)	50/104 (48%)	50/73 (68%)
Unselected (100)	30/89 (34%)	38/69 (55%)	42/94 (45%)	10/100 (10%)	1/100 (1%)	N/D	N/D

Numbers of cancers with that molecular change as proportion of total cancers of that type studied (and percentages) are shown. Pairwise and overall comparisons of each molecular variable between Series showed no significantly different frequencies (at p=0.05), except as detailed in the text. N/D = not determined. IHC = immunohistochemistry.

Table 2. Association between APC LOH and 18q LOH in FCC CRC Series

The table shows numbers of FCC cancers with LOH at the *APC* locus and close to the *SMAD4* locus on chromosome 18q21.1.

		APC		
		LOH	No LOH	Total
18q	LOH	24	12	36
	No LOH	16	30	46
Total		40	42	82

Figure 8.1 Results of Hierarchical Cluster Analysis on FCC Patients Split into Four Sets
Each branch of the dendrogram represents one family/patient. The Group (1 or 2) assigned by STATA *cluster group* command is shown below each branch of the dendrogram. For all four dendrograms, the cancers of those patients/families in Group 1 almost all had no K-ras mutation and those in group 2 almost all had a mutation. Five discordant families are shown marked by an asterisk.

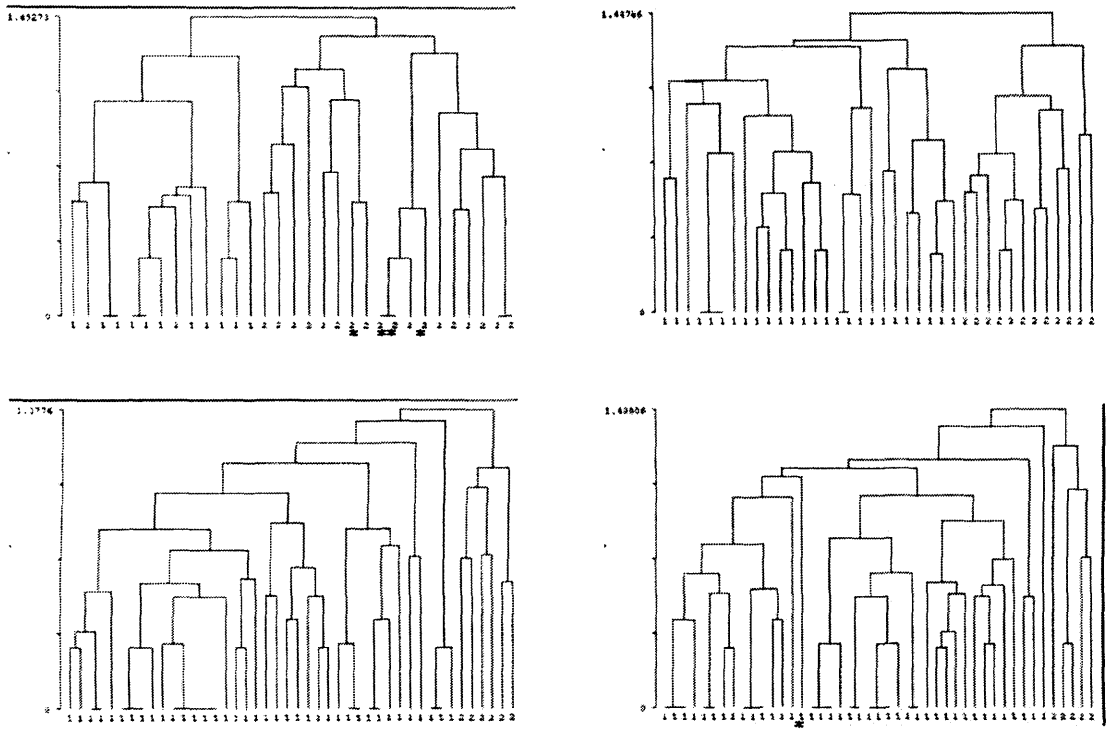

Figure 8. 1

8.5 References

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Chapter 9 Conclusions

Throughout the period of this thesis I have attempted to uncover information and explore themes relevant to familial colorectal cancer and polyposis. The project has spanned the genetics of FAP, HNPCC, MAP, and multiple colorectal adenomas of unknown cause. It has dealt with analysis of family history, case finding, associated cancer risks and mechanisms of tumourigenesis. I have been fortunate to be able to perform true transitional research in that the patients whom I saw and helped to manage in the familial cancer clinic contributed and in many cases ultimately derived some benefit from the research.

The first project undertaken was the classification of families with a strong history of colorectal cancers into strata, aiming to further analyse these groups, this was done by means of a cluster analysis. One of its novelties lay in the fact that families, individuals, cancer pathology and genetic information were analysed together, mimicking our true situation in the clinic. A broad range of information was collected about families including patterns of inheritance, cancer spectrum and ages at diagnosis as well as tumour pathology. As much molecular information s possible was also uncovered using techniques to detect gene mutations and losses and measure gene expression. The major problems encountered in this project were difficulties in fully verifying pedigrees as reported by probands, obtaining archival tumour samples from a range of hospitals from up to twenty years ago and working with DNA derived from paraffin-embedded tissue which may give poor results in mutation detection.

Despites this, 250 families were informative enough to be included in this analysis. The success in recruiting patients may largely be attributable to the existence of the Bobby Moore database at St Marks Hospital which is a comprehensive database of all patients seen and includes pathological and follow-up data. Many of the families with mutation positive HNPCC were diagnosed as such during this project after findings on tumours of MSI-H or MMR protein loss. Our methods of distinguishing such patients appeared to work well as very few families outside of these were likely to harbour mutations based on our cluster analysis. We saw little evidence for a discriminatory effect of TP53, beta-catenin, APC or SMAD changes between subgroups of colorectal cancers. There did appear to be two groups identifiable based on K-ras mutations. K-ras mutations are one of the most frequent events in colorectal adenoma and carcinoma development and this effect may be used as an initial

stratification in future studies of this nature. Key limitations in this study include sample size, and perhaps number of genes assessed.

Closely allied to such efforts in stratification of familial colorectal cancers is the issue of deciding upon which families have HNPCC with a germline mutation in a MMR gene. It is not always possible to carry out mutation testing in an effected individual to inform the family of overall risk and allow predictive testing to be undertaken. It is important to make the diagnosis of HNPCC in the right families to allow frequent screening colonoscopy even in the absence of germline mutation. Equally we wish to avoid over-screening families unlikely to carry a mutation as colonoscopy is not without risk of morbidity and mortality. In a recent paper, Lindor et al showed that unaffected relatives in families who met the Amsterdam criteria but did not harbour MMR gene mutations were at much lower risk for colorectal and other HNPCC-associated cancers than were those from mutation positive families (Lindor, Rabe et al. 2005). This finding certainly provided evidence that familial clustering may occur due to shared environmental or lifestyle influences or because of multiple low to moderate disease causing alleles within families.

We did improve upon the Amsterdam Criteria in terms of specificity and the Bethesda Criteria in terms of specificity using a simple algorithm which relies on pedigree information. To verify this we used groups of families where HNPCC status was known and then validated this in a test set where results of mutation detection were known. We proposed a method of classifying families as HNPCC even if no live affected member is available for mutation testing. This involves assessment of as many tumours are available and performing IHC for MMR proteins as well as MSI. Using our methods we define families who should go forward for further testing with MSI and IHC, with potential mutation testing in some. All families classified as HNPCC by our criteria should undergo regular (two yearly) colonoscopy with gynaecological screening for women.

In parallel with the above projects was the work performed on the syndrome of multiple colorectal adenomas. It had been recognised for some time that the majority of individuals who developed 5 to 100 colorectal polyps during their lifetime did not have mutations in the main gene known to cause a polyposis syndrome – *APC* nor were they part of the spectrum of HNPCC (Lamlum, Al Tassan et al. 2000). Many cases appear isolated or recessive and phenotypes vary widely. Our first effort in finding the genetic basis of such persons was a

candidate gene search in Wnt and SMAD pathway genes. A highly representative group of multiple adenoma patients were assessed and no pathogenic mutations were found in Wnt pathway genes in particular. This resounding negative result was somewhat surprising to us but convinced us that *APC* changes and effects from outside the Wnt pathway on beta-catenin may well be the major mechanisms of tumourigenesis here.

After the first report emerged of a group of siblings with germline mutations in *MYH* causing multiple adenomas in a seemingly recessive pattern we explored this mechanism in two large groups of patients, the first will multiple adenomas and the second with classical polypsis; both groups without detectible mutations in *APC*. We looked for mutations in *MYH* and also in two other important BER genes, *OGG1* and *MTH1*. Although we found no mutations in the latter two genes we confirmed that in around 30% of *APC* mutation negative patients with over 15 adenomas, bi-allelic *MYH* mutations were responsible for disease. We uncovered a number of new mutations some of which were likely to be pathogenic and confirmed that these patients have on average a higher number of adenomas than multiple adenoma patients without mutations. We also saw an excess of G→T transversion mutations in *APC* in tumours from bi-allelic mutation carriers. This along with the occurrence of MAP patients with duodenal polyposis made us hypothesis that APC dysfunction was a crucial step in the carcinogenic effect of *MYH* mutations.

Since our publication in 2003 a multitude of investigators have confirmed our findings in groups of multiple adenoma and polyposis patients from around the world (Fearnhead 2003; Hes F. 2003; Sampson, Dolwani et al. 2003; Sieber, Lipton et al. 2003; Gismondi, Meta et al. 2004; Kambara, Whitehall et al. 2004; Wang, Baudhuin et al. 2004; Miyaki, Iijima et al. 2005; Nielsen, Franken et al. 2005). It has become apparent also that founder mutations exist in certain ethnic populations with many others still unstudied.

The magnitude of colorectal cancer risk to the *MYH* heterozygote remains open to debate although recent studies put the relative risk at somewhere between 1.5 and three (Tenesa, Farrington et al. 2005; Jenkins, Croitoru et al. 2006). The implication is that mono-allelic *MYH* mutations may be low to moderate risk alleles for colorectal cancer. Several studies have looked for *MYH* mutations in cohorts of colorectal cancer patients in groups of sporadic colorectal cancer patients and some have shown an increased number of mono-allelic *MYH* mutation carriers compared to their population (Enholm, Hienonen et al. 2003; Fleischmann,

Peto et al. 2004; Kambara, Whitehall et al. 2004). Bi-allelic mutation carriers are also seen with few or no colorectal adenomas (Enholm, Hienonen et al. 2003; Wang, Baudhuin et al. 2004; Zhou, Djureinovic et al. 2005). If single *MYH* mutations do confer a degree of risk sufficient to warrant screening colonoscopy, the resource issues of such may be enormous depending entirely on the population frequency of *MYH* mutations. The population frequency of mutations has been estimated in a number of different populations with estimates between 0% and 1.8% for the Y165C mutations and between 0% and 2.2% for G382D mutations (Al-Tassan, Chmiel et al. 2002; Enholm, Hienonen et al. 2003; Sieber, Lipton et al. 2003; Fleischmann, Peto et al. 2004; Isidro, Laranjeira et al. 2004; Kambara, Whitehall et al. 2004; Wang, Baudhuin et al. 2004; Leite, Isidro et al. 2005; Zhou, Djureinovic et al. 2005). The frequency of potential founder mutations in other ethnic groups is unknown. As time progresses increasing numbers of MYH missense mutations are being found to be pathogenic also (Bai, Jones et al. 2005). The potential for *MYH* mutations to cause disease outside the gastrointestinal tract has not yet adequately been explored and is a fertile area for further research.

Mutations in the BER pathway are a novel initiating event in tumourigenesis and might have been expected to cause cancer in a way analogous to defective DNA mismatch repair. I performed the first exploration of this hypothesis in as many cancers and adenomas from biallelic MYH mutation carriers as possible. Because this is a rare syndrome and many patients were diagnosed retrospectively such material was not easy to come by, nonetheless around 20 cancers and 150 adenomas were able to be included in the analysis. The findings were most interesting and established beyond doubt that these tumours do not show MSI and that they are highly enriched for one specific K-ras mutation (G12C) both of which findings have since been confirmed by other investigators (Jones, Lambert et al. 2004; Kambara, Whitehall et al. 2004). My findings pointed strongly towards these tumours showing little gain or loss of genetic material however a recent publication by Cardoso et al using comparative genomic hybridisation has suggested that these tumours do show chromosomal instability to the same extent as FAP cancers (Cardoso, Molenaar et al. 2006).

Thus, a small but significant part of the problem of multiple colorectal adenomas has been solved by the description of MAP. Many other individuals and families exist at present for whom we cannot find a genetic explanation for disease. Methods worthy of consideration for further studies include the use of gene expression microarrays using tumour material, SNP

analysis of germline and tumour DNA and classical linkage analysis. Study of adenoma material is particularly important as 'initiating' events may be best appreciated at an early stage of tumourigenesis. Tumour and germline DNA banking is thus a crucial component of the ongoing progress towards explaining not only the genetic basis of colorectal cancer but how we can use this to prevent disease and better care for our patients.

Although we classically think of deleterious germline mutations in a small number of genes being responsible for inherited CRC, the truth is likely to be much more complex. It is probable that a range of mutations or polymorphisms in a range of genes may lead to a small increase in lifetime colorectal cancer risk (low to moderate penetrance alleles). Possibly if a number of these changes are inherited together, an individual or family may mistakenly be assumed to be carrying a mutation in a high penetrance cancer causing allele. Such variants may be common, such as *MYH* missense mutations (1-2% of the population), or extremely rare. Missense mutations in genes such *APC* and the MMR genes which have been thought to be non-pathogenic may in fact confer a small increase in risk, such that disease 'skipping' generations is common. Common variants conferring a mild increase in CRC risk acting on their own or in concert with other low penetrance alleles will only be appreciated using large studies, both case or cohort control and based on linkage, using families with apparent increase in CRC incidence. The CORGI study is an international research effort which will recruit over one thousand colorectal cancer families without known genetic predisposition and use a linkage approach to uncover new alleles involved in colorectal cancer risk. Large studies which follow cohorts of thousands of persons for many years may be used as cohorts for case control studies. Until we characterise the range of variants that lead to inherited forms of colorectal cancer it will remain difficult to give appropriate advice to the majority of patients who seek our advice in the Familial Colorectal Cancer Clinic.

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